UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
Departamento de Zoología y Antropología Física

TESIS DOCTORAL

Promiscuidad en la estrategia de reproducción del estornino negro : causas y consecuencias

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Elena García González

DIRECTORES:

Pedro Javier, dir Cordero Tapia
José Pablo, dir Veiga Relea

Madrid, 2015

Promiscuidad en la estrategia de reproducción del Estornino Negro: Causas y consecuencias

TESIS DOCTORAL

Elena García González

Universidad Complutense de Madrid
Facultad de Ciencias Biológicas
Departamento de Zoología y Antropología Física

Septiembre de 2011

Universidad Complutense de Madrid

Facultad de Ciencias Biológicas

Departamento de Zoología y
Antropología Física

Promiscuidad en la estrategia de reproducción del Estornino Negro: Causas y consecuencias

Memoria presentada por Elena García González para optar al grado de Doctora en Ciencias Biológicas

La Doctoranda

Elena García González

V° B° de los Directores

Pedro Javier Cordero Tapia

José Pablo Veiga Relea

Septiembre de 2011

Esta tesis ha sido financiada por los proyectos de investigación: CGL-2005- 05611-C02-01 y 02/BOS (Ministerio de Ciencia e Innovación), CGL2008-00095/BOS (Ministerio de Ciencia e Innovación), PCI08-0130 (Junta de Comunidades de Castilla- La Mancha), POII10-0197-0167(Junta de Comunidades de Castilla- La Mancha), PEII11-0161-4455 (Junta de Comunidades de Castilla- La Mancha) y los fondos 2005-SGR-00090 (Direcció General de Recerca de la Generalitat de Catalunya). Durante la realización de esta tesis disfruté de una beca predoctoral de formación de profesorado universitario (FPU) del Ministerio de Educación y Ciencia.

A mis padres y a Edu

ÍNDICE

AGRADECIMIENTOS

Hoy, como tantos otros días sentada frente al ordenador y en una tarde en la que precisamente comienza el verano, me parece increíble enfrentarme por fin a la escritura de esta página, la página más ansiada por cualquiera que se embarque en la ardua tarea de escribir una tesis doctoral, ya que supone el paso final para cerrar, por fin, ese pequeño libro que tantos quebraderos de cabeza ha conllevado.

Ahora, al echar la vista atrás, recuerdo como a tan sólo unos metros del lugar en el que me encuentro, daba saltos de alegría al enterarme de que tenía la oportunidad de empezar esta nueva aventura. Por mi mente pasan todas esas personas que de un modo u otro han influido en que haya logrado llegar a escribir estas líneas.

En primer lugar me gustaría agradecer a mis directores Pedro y Pablo, por darme la posibilidad de entrar en este mundo, por compartir conmigo su espacio, su sabiduría y su experiencia ¡y por llevarme a Cuba! pero en especial a Pablo por la libertad que me otorgó y su infinita paciencia y a Pedro por estar siempre al otro lado, porque aunque me ausentase o fuese imposible contactar conmigo por teléfono, no perdió su esperanza en mí, incluso cuando yo ya lo había hecho. También a los dos, gracias por ser tan diferentes, lo que al final (quién me lo iba a decir...), ha resultado tan enriquecedor. También a Vicente, por enseñarme tantas curiosidades y por sus bromas que hacían el trabajo un poco más llevadero, aunque junto a Pablo, es el responsable por sus fotos de Kenia y sus historias de la Antártida, de que el suelo del MNCN tenga dos bonitas rayas dejadas por mis dientes...

José Miguel, Joaquín y Gustau no podía dejar de nombraros por ser mí otra familia investigadora. Por tratarme siempre tan bien y por las risas que nos hemos echado unas cuantas veces hablando acerca de temas de selección sexual en vertebrados. Pilar, ¡no has podido incorporarte en mejor equipo!

Como no, a mis compañeros, o mejor dicho amigos, del Museo, tanto a los que conocí desde el principio en el laboratorio como al resto que me adoptasteis como parte de vuestro grupo. ¡Cuantas comidas, alegrías, penas y confidencias hemos compartido a lo largo de este tiempo! Sería imposible agradecer uno por uno todo lo que os debo pero Laura: al final la gota perfora la roca ¡Gracias por ser todo un ejemplo!; Ana, Ángela, Mari Jose, Raquel, Lucia, Diego: ¡Gracias por aguantarme, entenderme y animarme siempre!; Fer, Carlos, Patri, Paula, Iván, Elena, Paty y Carlos tampoco me olvido de vosotros...¡Al final aquellas retahílas de tubos Eppendorf infinitas y los turnos de las 9 de la noche reservados para EGG han dado como resultado este "puñado de hojas"!. También a ti, Oscar, por tener tu puerta siempre abierta y escucharme aunque al día siguiente se te acabase el plazo para entregar un proyecto o revisar un artículo... ¡GRACIAS! Con otros, no tuve la oportunidad de compartir tantos momentos (las PCR y el SPSS absorben muuuucho tiempo) pero aun así, gracias por tener una sonrisa al cruzarnos en el pasillo y estar dispuestos a ayudar: Conchi... ¡que hubiera sido de la tramitación sin ti!

Un recuerdo especial para Gonzalo y Elena, quienes me atrevería a decir fueron determinantes en la decisión de eso de "querer ser Doctora", con vosotros aprendí y me divertí, a partes iguales, buscando diminutas flores "raras" en las laderas de las montañas por la geografía española, ¡eso sí que eran aventuras! Aumentasteis, en cierto modo, mis ganas de investigar, de saber, de conocer. Creo que fue con vosotros con los que más "Bióloga" me he sentido.

A Silvia, ¡Ánimo! Ahora veo que es posible. ¡Gracias por no dejarme caer nunca!, a Edu por ayudarme con muchas de las dudas burocráticas y al resto de amigos a los que eso de estudiar como los estorninos elegían a sus parejas les parecía de lo más extraño y divertido: Quique, Xua, Yoly, Vero, Lore, Celia, Paula....y a Ursu también por sus sutilezas (tu me entiendes...). A los que pasaron por mi vida y nunca han dejado de confiar en mí, lo del Nobel va a ser que lo dejamos... (Tú sabes que me refiero a ti).

Por último, pero no por ello menos importante a mi familia, por ser el pilar básico de mi vida, por respetar, aunque no lo entendierais, a esta oveja negra que se alejó de las Ciencias Económicas para irse a otras ciudades a estudiar "Bichos y Flores", por quererme, por animarme siempre, por dejarme seguir mi camino, porque nunca me habéis fallado, por no haberme presionado, (salvo con el "¡Queremos ir a Madrid ya!") y sobre todo por haberme dejado arriesgar....

A Edu… creo que no tendría hojas suficientes, aunque dedicase una tesis completa sólo a eso, para agradecerte todo lo que has hecho por mí durante todos estos años. De hecho, creo que un trocito de esta tesis te pertenece, no sólo por toda la ayuda técnica con el Word (¡dichosos tabuladores!), las imprentas, o porque incluso hasta el ordenador desde el que escribo es tuyo, sino por haber hecho posible que lo consiga. Sin ti, nunca habría podido. Así que GRACIAS. Ahora sí, ya podemos empezar a soñar…

INTRODUCCIÓN GENERAL

Los patrones de comportamiento sexual que se observan en los animales son el resultado de presiones selectivas que actúan diferencialmente sobre machos y hembras. En unos casos, estas presiones pueden tener la misma dirección (lo que es bueno para un sexo también lo es para el otro) pero, en general, tienen direcciones opuestas (Carranza 1994). Así, las estrategias reproductoras que acaban por darse finalmente en una especie dependen del balance individual entre costes y beneficios de llevar a cabo una u otra estrategia y de los diferentes intereses de machos y hembras (Orians 1971; Alatalo *et al.* 1981; Oring 1982; Davies 1989; Clutton-Brock 1991; Moreno *et al.* 2002). Por lo tanto, entre poblaciones, o incluso dentro de la misma población, pueden coexistir múltiples estrategias de apareamiento (Owens & Bennet 1997) o, lo que es lo mismo, existe una variabilidad intraespecífica en la estrategia de reproducción óptima en función de las características de los individuos o de las condiciones ambientales presentes en ese momento. Puede haber individuos que no presenten las características necesarias para desarrollar la estrategia de apareamiento óptima, por ejemplo, individuos que no sean capaces o no tengan aún la edad necesaria para defender un territorio. Sea cual sea el caso, esos individuos se verán obligados a adoptar estrategias de reproducción que tendrán menos beneficios que las estrategias principales, pero que al menos les permitirán reproducirse. Esto es lo que se entiende en Ecología del comportamiento como "El mejor de un mal oficio" ("The Best of a Bad Job") y es, por ejemplo, el caso de los machos satélite o flotantes de numerosas especies de diferentes taxa que defienden harenes o territorios, como la rana toro americana (*Rana catesbiana;* Howard 1978), el sapo corredor (*Bufo calamita*; Arak 1988) o el elefante marino (*Mirounga angustirostris*; Le Boeuf 1974). En otras situaciones puede tratarse de individuos que, por azar o limitación de parejas están apareados con individuos de baja calidad y llevan a cabo cópulas extra-pareja (EPCs) con el fin de intentar aumentar su éxito reproductor. Incluso un mismo animal puede elegir distintas estrategias dependiendo de condiciones concretas, como se ha demostrado por ejemplo en el acentor común (*Prunella modularis).* En esta especie el sistema puede cambiar de poliginia a poliandria en función de la capacidad del macho para monopolizar a las hembras, que a su vez dependerá de la abundancia de comida en el territorio. Así, cuando la comida es abundante las hembras no necesitan desplazarse demasiado para encontrar alimento y es posible que un solo macho pueda controlar varias hembras y acapararlas dando lugar a sistemas de apareamiento poligínicos y poliginándricos. Mientras que, cuando la comida es escasa, las hembras necesitan desplazarse mucho más para encontrar alimento, haciendo difícil para los machos su monopolización y dando lugar a sistemas poliándricos (Davies y Lundberg 1984).

Debemos también tener en cuenta que el éxito de una estrategia depende de las utilizadas por los demás individuos. Es decir, la estrategia de algunos individuos de comportarse como machos satélite no tendría sentido si no existiesen machos territoriales. Incluso si el número de machos territoriales fuese muy alto con respecto al de satélites, podría ocurrir que, en promedio, ambas estrategias resultaran igualmente exitosas. Cuando se establece un equilibrio en el número de individuos que adoptan cada estrategia, de modo que los beneficios asociados a cada una de ellas se igualan, se suele hablar de estrategias alternativas en equilibrio evolutivo o simplemente de estrategias alternativas. Estas estrategias pueden tener base genética, como ocurre en el salmón coho (*Oncorhynchus kisutch,* Gross 1985) y en el pez sol (*Lepomis macrochirus*, Gross y Charnov 1980), o no, como en la avispa cavadora (*Sphex ichneumoneus*, Brockmann *et al.* 1979).

La información hasta ahora disponible sugiere que entre los machos las EPCs son parte de una estrategia reproductora mixta (Trivers 1972; Beecher y Beecher 1979; Fitch y Shugart 1984), más que un comportamiento reproductor especializado adoptado exclusivamente por un subgrupo de la población. Es más, en muchas especies, los machos implicados en las EPCs son normalmente residentes en territorios vecinos y están ya apareados. Por ejemplo, un estudio en el ibis blanco americano (*Eudocimus albus*) mostró que los machos que buscaron EPCs eran machos que defendían nidos vecinos, no machos de zonas alejadas de la colonia o que no tuvieran un nido (Frederic 1987). La vecindad como correlato de las EPCs se ha observado también en garcilla bueyera (*Bulbucus ibis*) (Fujioka y Yamagishi 1981), arao común (*Uria aalge*) (Birkhead *et al.* 1985), fulmar boreal (*Fulmarus glacialis*) (Hatch 1987), papamoscas cerrojillo (*Ficedula* *hypoleuca*) (Alatalo *et al.* 1984), abejaruco (*Melops bullockoides*) (Emlen y Wrege 1986), escribano azul (*Passerina cyanea*) (Westneat 1987a, b); oropéndola de Baltimore (*Icterus galbula*) (Edinger 1988), diamante mandarín (*Taeniopygia guttata*) (Birkhead *et al.* 1988) y perdiz nival (*Lagopus mutus*) (Brodsky 1988). Está menos claro si las EPCs son parte de una estrategia mixta entre las hembras. Por un lado, las hembras apareadas a veces buscan y solicitan EPCs como en el fulmar boreal (Hatch 1987) y carbonero sibilino (*Parus atricapillus*) (Smith 1988). Por otro lado, muchos estudios han documentado que las EPCs son vigorosamente resistidas por las hembras (McKinney *et al.* 1984). Sin embargo, incluso en esos casos, la implicación de las hembras en las EPCs podría ser parte de una estrategia de apareamiento mixta que aún no es bien conocida.

Lo que si está claro es que las EPCs están mucho más ampliamente extendidas en las aves de lo que se pensaba en un principio. De hecho, al contrario de lo que proponía Lack en 1968, la verdadera monogamia ocurre en menos del 25% de las especies socialmente monógamas y la paternidad extra-pareja (EPP), entendida en esta tesis como la presencia de pollos que son hijos genéticos de la hembra social pero no del macho social de un nido, se da de manera regular en el 75% de las especies restantes (Griffith *et al.* 2002). Además de las EPCs, existen otros tipos de relaciones extra-pareja como el pseudoparasitismo, en el que una hembra se aparea con un macho ya emparejado de la misma especie y pone sus huevos en el nido de éste, o el parasitismo intraespecífico, en el que una hembra pone sus huevos de forma parásita en otros nidos. Ambas estrategias pueden llevar a la aparición en los nidos de pollos no relacionados genéticamente, bien con sólo uno de los dos padres sociales o con ambos. De forma general, podemos considerar todas estas estrategias como conductas potencialmente promiscuas, ya que aunque no necesariamente implican el apareamiento con más de un individuo del sexo opuesto, sí implican emparejamientos fuera de las parejas establecidas socialmente.

La cuestión acerca de las razones del éxito de las estrategias promiscuas, y en especial de las EPCs, ha sido tema de estudio a lo largo de las últimas décadas. Así, por ejemplo, la variación en las tasas de EPP ha sido relacionada con factores demográficos como la sincronía de cría, que puede relacionarse positivamente con las tasas de EPP (Stutchbury y Morton 1995), o la necesidad de cuidado parental por parte del macho, que está a menudo correlacionada con reducidas tasas de EPP (Ver Capítulo 4 de esta tesis). Sin embargo, la verdadera función adaptativa de las estrategias promiscuas para las aves es un tema aún pendiente a pesar de los numerosos trabajos que han abordado este tema (revisado en Griffith *et al.* 2002). El beneficio de estas estrategias parece claro desde el punto de vista de los machos que consiguen aumentar su éxito reproductor, fertilizando una hembra a un pequeño coste, a expensas de otro macho que será el que proporcionará el cuidado parental. Para las hembras, sin embargo, los beneficios no son tan claros y se han desarrollado varias hipótesis para explicar por qué puede ser ventajoso para ellas adoptar este tipo de estrategias promiscuas. Los argumentos apuntan a que las hembras podrían obtener beneficios ecológicos directos (Gray 1997; Blomqvist *et al.* 2005) o, más probablemente, beneficios genéticos indirectos en su descendencia derivados de los emparejamientos extra-pareja (Birkhead y Møller 1992; Petrie y Kempenaers 1998; Petrie *et al.* 1998; Jennions y Petrie 2000; Griffith *et al.* 2002). Dentro de este tipo de hipótesis se englobarían la hipótesis de la diversidad genética (Williams 1975; Westneat *et al.* 1990), la hipótesis de garantía de la fertilidad (Wetton y Parkin, 1991; Sheldon 1994), la hipótesis de los "buenos genes" (Møller 1988; Hamilton 1990; Westneat *et al.* 1990; Birkhead y Møller, 1992) y la hipótesis de compatibilidad genética (Kempenaers *et al.* 1999; Tregenza y Wedell 2000) (Ver Capítulo 5 de la presente tesis). Todas las hipótesis basadas en la obtención de beneficios genéticos indirectos por parte de la hembra como resultado de emparejamientos promiscuos contemplan en sus predicciones la presencia o ausencia de diferencias en la calidad de los individuos y por tanto la presencia o ausencia de diferencias fenotípicas o genéticas entre los machos sociales y los machos extra-pareja. La evaluación de la calidad de los individuos mediante caracteres fenotípicos o genéticos es, por tanto, un aspecto clave a analizar a la hora de arrojar luz sobre la posible función adaptativa de la EPP en una especie (Ver Capítulos 3 y 5 de la presente tesis). Por ejemplo, si consideramos que las hembras buscan como machos extra-pareja aquellos de mejor calidad que su pareja social no sería raro esperar que los machos desarrollasen caracteres indicadores de su calidad genética, como pueden ser los ornamentos, en respuesta a la selección sexual por parte de las hembras (Møller y Birkhead 1994). En este sentido, la teoría también predice que aquellos machos más ornamentados tienen un mayor éxito reproductor. Así, el lazo de unión entre señalización-selección sexual y éxito reproductor vendría dado por una estrecha relación entre eficiencia en la fertilización y expresión fenotípica de los ornamentos (Sheldon 1994).

Finalmente, aunque se ha trabajado intensamente en ello, no está del todo claro si las conductas promiscuas tienen ventajas selectivas y por tanto podrían perpetuarse como estrategias evolutivas que aumentan la eficacia biológica de los individuos que las practican. Es por tanto de gran interés conocer los patrones de fertilización extra-pareja en las poblaciones naturales, especialmente qué individuos son los que desarrollan estas estrategias y qué factores las pueden afectar. Además, el estudio de otras estrategias de reproducción, potencialmente relacionadas con la promiscuidad, como el pseudoparasitismo y el parasitismo intraespecífico puede ayudar a comprender procesos evolutivos tan complejos como la señalización, la selección sexual y la evolución de los sistemas de apareamiento.

BIBLIOGRAFÍA

Alatalo R.V, Carlson, A., Lundberg, A. & Ulfstrand, S. 1981. The conflict between male polygamy and female monogamy: the case of the Pied Flycatcher *Ficedula hypoleuca*. *American Naturalist*, **117**: 738-753.

Alatalo, R.V., Lundberg, A. & Stahlbrandt, K. 1984. Female mate choice in the pied flycatcher *Fycedula hypoleuca*. Behavioral Ecology and Sociobiology, **14**: 253-261.

Arak, A. 1988. Callers and satellites in the natterjack toad: Evolutionarily stable decision rules. *Animal Behaviour*, **36**: 416-432.

Beecher, M.D. & Beecher, I.M. 1979. Sociobiology of Bank Swallows: Reproductive strategy of the male. *Science*, **205**: 1282-1285.

Birkhead, T.R., Clarkson, K. & Zann, R. 1988. Extra-pair courtship, copulation and mate guarding in wild Zebra Finches *Taeniopygia guttata. Animal Behaviour*, **36**: 1853-1855.

Birkhead, T.R. & Møller, A.P. 1992. *Sperm competition in birds: evolutionary causes and consequences.* London: Academic Press.

Birkhead, T.R., Jonson, S.D. & Nettleship, D.N. 1985. Extra-pair mating and mate guarding in the Common Murre *Uria aalge*, *Animal Behaviour*, **33**: 608-619.

Blomqvist, D., Fessl, B., Hoi, H. & Kleindorfer, S. 2005. High frequency of extrapair fertilisations in the moustached warbler, a songbird with a variable breeding system. *Behaviour*, **142**: 1133-1148.

Brockmann, H.J., Grafen, A., & Dawkins, R. 1979. Evolutionarily stable nestling strategy in a digger wasp. *Journal of Theoretical Biology*, **77**: 473-496.

Brodsky, L. M. 1988. Mating tactics of male Rock Ptarmigan, *Lagopus mutus*: A conditional mating strategy, *Animal Behaviour*, **36**: 335-342.

Carranza, J. 1994. *Etología. Introducción a la Ciencia del Comportamiento*. Cáceres: Universidad de Extremadura.

Clutton-Brock, T. H. 1991. *The evolution of paternal care*. Princeton: Princeton University Press.

Davies, N. B. 1989. Sexual confict and the polygamy threshold. *Animal Behaviour*, **38**: 226-233.

Davies, N. B. & Lundberg, A. 1984. Food distribution and a variable mating system in the dunnock (*Prunella modularis*). *Journal of Animal Ecology*, **53**: 895-912.

Edinger, B. B. 1988. Extra-pair courtship and copulation attempts in Northern Orioles, *Condor*, **90**: 546-554.

Emlen, S. T. & Wrege, P. H. 1986. Forced copulations and intraspecific parasitism: Two costs of social living in the White-fronted Bee-eater. *Ethology,* **71**: 2-29.

Fitch, M. A. & Shugart, G. W. 1984. Requirements for a mixed reproductive strategy in avian species. *American Naturalist*, **124**: 116-126.

Frederick, P. C. 1987. Extra-pair copulations in the mating system of White Ibis (*Eudocimus albus*). *Behaviour*, **100**: 170-201.

Fujioka, M. & Yamagishi, S. 1981. Extramarital and pair copulation in the Cattle Egret. *Auk*, **98**: 134-144.

Gray, E. M. 1997. Female red-winged blackbirds accrue material benefits from copulating with extra-pair males. *Animal Behaviour*, **53**: 625–639.

Griffith, S. C., Owens, I. P. F. & Thuman, K. A. 2002. Extra-pair paternity in birds. A review of interspecific variation and adaptative function. *Molecular Ecology*, **11**: 2195-2212.

Gross, M. R. 1985. Disruptive selection for alternative life histories in salmon. *Nature*, **313**: 47-48.

Gross, M. R. & Charnov, E. L. 1980. Alternative male life histories in bluegill sunfish. *Proceedings of the National Academy of Sciences of U.S.A,* **77**: 6937-6940.

Hamilton, W.D. 1990. Mate choice near and far. *American Zoologist*, **30**: 341-352.

Hatch, S. A. 1987. Copulation and mate guarding in the Northern Fulmar. *Auk*, **104**: 450-461.

Howard, R. D. 1978. The evolution of mating strategies in bullfrogs, *Rana catesbiana*. *Evolution*, **32**: 850-871.

Kempenaers, B., Congdon. B., Boag, P. & Robertson, R.J. 1999. Extrapair paternity and egg hatchability in tree swallows: evidence for the genetic compatibility hypothesis? *Behavioral Ecology*, **10**: 304-311.

Jennions, M. D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews*. **75**: 21-64.

Lack, D. 1968. *Ecological Adaptations for Breeding in Birds*. London: Methuen.

Le Boeuf, B. J. 1974. Male-male competition and reproductive success in elephant seals. *American Zoologist*, **14**: 163-176.

McKinney, F., Cheng, K. M. & Bruggers, D. J. 1984. Sperm competition in apparently monogamous birds. In: *Sperm Competition and the Evolution of Animal Mating Systems* (Ed. by R.L. Smith), pp 523-545. Orlando: Academic Press.

Møller, A. P. 1988. Female choice selects for male sexual tail ornaments in the monogamous swallow. *Nature*, **332**: 640-642.

Møller, A. P. & Birkhead, T. R. 1994. The evolution of plumage brightness in birds is related to extra-pair paternity. *Evolution*, **48**: 1089-1100.

Moreno J., Veiga, J. P., Romasanta, M. & Sánchez, S. 2002. Effects of maternal quality and mating status on female reproductive success in the polygynous spotless starling. *Animal Behaviour*, **64**: 197-206.

Orians, G. H. 1971. Ecological aspects of behaviour. In: *Avian Biology*. Vol. I (Ed. by D.S. Farner & J.R. King), pp. 513-546. New York: Academic Press.

Oring, L. W. 1982. Avian mating systems. In: *Avian Biology*. Vol. VI. (Ed. by D.S. Farner, J.R. King & K.C. Parkes), pp. 1-92. New York: Academic Press.

Owens I. P. F. & Bennet, P. M. 1997. Variation in mating system among birds: ecological basis revealed by hierarchical comparative analysis. *Proceedings of the Royal Society of London, Series B*, **264**: 1103-1110.

Petrie, M., Doums, C. & Møller, A. P. 1998. The degree of extra-pair paternity increases with genetic variability. *Proceedings of the National Academy of Sciencies of U.S.A.*, **95**: 9390-9395.

Petrie, M. & Kempenaers, B. 1998. Extra-pair paternity in birds: explaining variation between species and populations. *Trends in Ecology & Evolution*, **13**: 52-57.

Sheldon, B. C. 1994. Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds. *Proceedings of the Royal Society of London, Series B*, **257**: 25-30.

Smith, S. M. 1988. extra-pair copulations in Black-capped Chickadees: The role of the female. *Behaviour*, **107**: 15-23.

Stutchbury, B. J. & Morton, E. S. 1995. The effects of breeding synchrony on extra-pair mating systems in songbirds. *Behaviour,* **132**: 675-690.

Tregenza, T. & Wedell, N. 2000. Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology*, **9**: 1013–1027.

Trivers, R. L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (Ed. by B.G. Campbell), pp. 136-179. Chicago: Aldine.

Westneat, D. F. 1987a. Extra-pair fertilizations in a predominantly monogamous bird: genetic evidence. *Animal Behaviour,* **35**: 877-886.

Westneat, D. F. 1987b. Extra-pair copulations in a predominantly monogamous bird: Observations of behaviour. *Animal Behaviour*, **35**: 865-876.

Westneat, D.E., Sherman, P.W. & Morton, M.L. 1990. The ecology and evolution of extra-pair copulations in birds: In: *Current Ornithology,* Vol. 7, pp 331-369 (Ed. by D.M. Power) New York: Plenum Press.

Wetton, J.H. & Parkin, D.T. 1991. An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. Proceedings of the Royal Society Series B, **245**: 227-233.

Williams, G.C. 1975. *Sex and evolution*. Princeton: Princeton University Press.

METODOLOGÍA GENERAL

1. Especie y área de estudio

El estornino negro (*Sturnus unicolor*) es un paseriforme de la familia *Sturnidae*, de tamaño medio (longitud: 21-23cm, envergadura: 38-42cm), colonial y sedentario. En la actualidad y tras expandir su área de distribución a lo largo de las últimas décadas (Ferrer *et al.* 1991), ocupa la mayor parte de la Península Ibérica aunque es accidental en el archipiélago de las islas Canarias y en el archipiélago de las islas Baleares, salvo en la isla de Menorca donde puede considerarse escaso (Martín y Lorenzo 2001). Ocupa también una estrecha franja en el norte de África y las islas de Córcega, Cerdeña y Sicilia. Altitudinalmente, se distribuye entre 0 y 1500 m.s.n.m. Por encima de esta cota es raro, en parte por la falta de hábitat de nidificación adecuado (Veiga y Polo 2003).

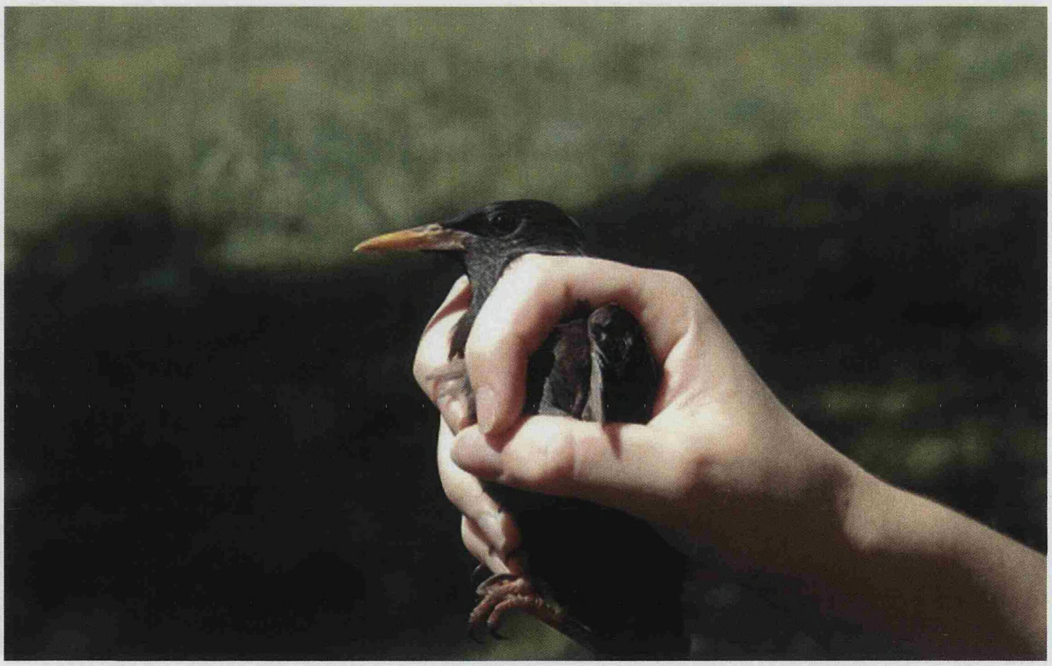

Foto 1. Estornino negro. Hembra adulta.

Se trata de una especie con cierto dimorfismo sexual. Los machos adultos son aproximadamente un 6% más pesados y tienen las alas un 3% más largas que las hembras (Cordero *et al.* 2001). No obstante, el empleo exclusivo de caracteres morfológicos biométricos no es aconsejable para identificar sexos en esta especie ya que casi se identifica erróneamente un individuo de cada cuatro, mientras que el empleo de caracteres morfológicos cualitativos es más fiable, con márgenes de error no mayores al 5 % (Peris 1989). En general, los adultos de ambos sexos pueden diferenciarse por la tonalidad y lustrosidad de su plumaje, que es más negro e iridiscente en los machos, y por la tonalidad de su iris, que en los machos es pardo oscuro mientras que en las hembras puede presentar un anillo exterior de color más claro (Veiga y Polo 2003), aunque este carácter no es del todo concluyente, ya que existen hembras con iris de un color uniforme. En época de reproducción el color de la base del pico constituye un buen carácter diferenciador entre ambos sexos ya que en los machos es gris-azulada y en las hembras rosada, mientras que en invierno ambos sexos la tienen negra u oscura (Veiga y Polo 2003).

Fotos 2 y 3. Dimorfismo sexual en adultos de estornino negro (*Sturnus unicolor*). Se observan claramente las diferencias en el color de la base del pico y del iris. Fuente: http://www.ibercajalav.net/img/417_*Sturnus_unicolor*.pdf. Foto 2: macho; Foto 3: hembra.

Tanto machos como hembras presentan plumas en la garganta y pecho cuya longitud tiende a incrementarse con la edad (Veiga y Polo, datos no publicados). En los machos estas plumas, cuya longitud ha sido relacionada con su nivel de heterocigosidad, son un 41% más largas que en las hembras (Aparicio *et al.* 2001) y se estrechan abruptamente desde la mitad hasta la punta, mientras que en las hembras este estrechamiento es mucho más gradual (Hiraldo y Herrera 1974; Lezana *et al.* 2000).

En los juveniles la identificación del sexo sobre la base de caracteres morfológicos es algo más complicada. Ambos sexos presentan el plumaje y las patas de color pardo oscuro y el pico oscuro. Después de la primera muda tienen los ápices de las plumas del cuerpo y la cabeza de color blanquecino pudiéndose confundir entonces con el estornino pinto (*Sturnus vulgaris*) con plumaje parcialmente desgastado. No es de extrañar, por tanto, que para la identificación del sexo de los pollos lo más apropiado sea recurrir al sexado mediante técnicas moleculares. En concreto los marcadores de ADN permiten sexar pollos de muy corta edad y proporcionan un método sencillo, rápido y fiable de determinación sexual a partir de las diferencias específicas en algunas secuencias del genoma que presentan los dos sexos. La técnica se basa en la amplificación mediante la técnica de PCR (Reacción en Cadena de la Polimerasa) y el empleo cebadores específicos de una secuencia interna (intrón) del gen de la proteína cromo-helicasa de unión a ADN (proteína CHD) que se encuentra conservada en la mayoría de las aves en los cromosomas sexuales Z y W (Griffiths *et al.* 1998; Kahn *et al.* 1998; Fridolfsson y Ellegren 1999). Este segmento de ADN es en general más largo en el cromosoma W que en el cromosoma Z debido a la presencia de bases de ADN adicional (aunque ver Griffiths *et al.* 1998; Kahn *et al.* 1998) y se puede distinguir mediante una sencilla electroforesis en gel de agarosa. Dado que las hembras son heterogaméticas (presentan dos cromosomas sexuales distintos (ZW)) y que los machos son homogaméticos (ambos cromosomas sexuales son del mismo tipo (ZZ)), las bandas dobles corresponderán a individuos hembra y las bandas únicas a individuos macho.

Foto 4: Gel de electroforesis para el sexado de 4 individuos: 3 hembras y 1 macho. (Fuente: Universidad Complutense de Madrid)

Aunque se han diseñado muchos cebadores para llevar a cabo el sexado de las aves (Cerit y Avanus 2007), la pareja de cebadores más comúnmente empleada en paseriformes es P2 y P8 (Griffiths *et al.* 1998) que ha demostrado su utilidad en numerosas especies de aves (ej. Birkhead *et al.* 2001; Jensen *et al.* 2003). El sexado de aves mediante esta técnica es rápido y fiable y permite sexar pollos de muy corta edad (Griffiths *et al.* 1992).

La estrategia de reproducción más común en esta especie es la poliginia facultativa, en la que entre el 50% y el 85% de los machos adquiere dos o más hembras. La poliginia en los estorninos está limitada principalmente por el acceso a los nidos (Sandell y Smith 1996), especialmente cuando no hay un exceso en la disponibilidad de cajas y sólo unos pocos machos son capaces de controlar las que hay (Wittenberger 1976; Pintxen *et al.* 1989). No obstante, diferentes autores han mostrado la existencia de pollos extra-pareja, parásitos y pseudoparásitos en los nidos de esta especie (ver Calvo *et al.* 2000; Cordero *et al.* 2003), por lo que otras estrategias de reproducción están presentes en el estornino negro.

La edad más frecuente de primera reproducción es un año en las hembras y 2-3 en los machos y en ambos sexos la edad reproductora puede exceder los 7 años (Veiga y Polo 2003). Tanto los machos como las hembras defienden sus nidos frente a los competidores y la competición intra-sexual por los lugares de cría es dura en ambos sexos. La cría es mayoritariamente sincrónica. Las puestas en el área de estudio tienen lugar desde mediados de abril a mediados de junio y la mayoría de las hembras hacen dos puestas en cada estación de cría que constan comúnmente de 4-5 huevos de color azul incubados sobre todo por la hembra (Veiga y Polo 2003). La incubación dura entre 10 y 15 días desde la puesta del último huevo a la eclosión del primer pollo, con un valor modal de 11 días (Veiga y Polo 2003).

Foto 5: Huevos de estornino negro marcados en caja nido.

Los pollos eclosionados son alimentados por ambos padres, abandonan el nido entre los 18 y los 25 días de edad y continúan siendo alimentados por los adultos de 2 a 7 días más (Cramp y Perrins 1994). El éxito reproductor es muy variable entre localidades, entre años y entre primeras y segundas puestas. La mortalidad de los pollos varía del 21 al 58 % (Veiga *et al.* 2002) siendo la principal causa de mortalidad parcial de la pollada la falta de alimento para los pollos. La depredación por parte de urracas (*Pica pica*) y ratas (*Rattus sp*) es la principal causa de pérdida de polladas completas (Peris 1984, en Cramp y Perrins, 1994; Veiga *et al.* 2002). Así, se ha documentado la depredación de entre un 15 y un 48% de polladas enteras por parte de urracas cuando los pollos de estornino tienen el tamaño suficiente como para alcanzar la entrada del nido (Moreno *et al.* 1999, 2002). La media de pollos volados por hembra y año va de 5 en el centro de España a 6.1 en el nordeste (ver Peris 1984, en Cramp y Perrins 1994; Motis 1985, en Cramp y Perrins 1994; Pascual *et al.* 1992, en Cramp y Perrins 1994;

Veiga *et al.* 2002). El éxito de reclutamiento de los pollos en el año siguiente a su nacimiento en la misma zona de cría es muy escaso (López-Rul *et al.* 2011; observaciones personales de J.P. Veiga y V. Polo).

Foto 6: Pollos de estornino negro en caja nido

Las razones para elegir esta especie como modelo de estudio se basan principalmente en su abundancia, su facilidad para criar en cajas nido, y su estrategia de reproducción. Además, el estornino negro ha demostrado repetidamente su validez como especie modelo para el estudio de cuestiones fundamentales relacionadas con la biología reproductora de las aves. Así, se han hecho manipulaciones hormonales a nivel de huevo para ver sus efectos en el desarrollo y supervivencia de los pollos (Müller *et al.* 2007); a nivel del macho para ver su efecto en el cuidado parental y en el éxito reproductor (Moreno *et al.* 1999) o para evaluar los posibles costes de la poliginia (Cordero *et al.* 2003) y a nivel de hembras para ver cómo afecta a su rango social , al sex ratio de sus pollos (Veiga *et al.* 2004) o a su eficacia biológica a lo largo de su vida (Veiga y Polo 2008). Estos trabajos han demostrado que la manipulación hormonal es un método válido para el estudio de las estrategias reproductoras en aves. De hecho son muchos los autores que han usado esta especie como modelo para cuestiones relacionadas con la biología reproductora de las aves tales como las respuestas frente al parasitismo intraspecífico (Eens y Pinxten 1999), los efectos del status de la hembra sobre su éxito reproductor (Moreno *et al.* 2002), la función de los ornamentos como indicadores de calidad, ya sea considerando caracteres como las plumas (Cuervo y Møller 1999; Aparicio *et al.* 2001), el uso de plantas y plumas en los nidos (Polo y Veiga 2006; Veiga y Polo 2008) o la coloración de los huevos (López-Rul *et al.* 2008). Los pollos de esta especie han sido utilizados como modelo para, entre otros, el estudio de la función de petición en ausencia de los padres (Bulmer *et al.* 2008) y la competición entre hermanos (Gil *et al.* 2008). Además se dispone de una amplia base de datos de reproducción y morfométricos sobre la especie ya que la colonia de estudio se lleva monitorizando de manera continuada desde 1996. (Moreno *et al.* 1999; Cordero *et al.* 2001, 2003; Veiga *et al.* 2002).

El hecho de tratarse de una especie con poliginia facultativa supone una ventaja porque permite estudiar el éxito reproductor del macho *per sé*. Dado que un mismo macho se aparea con varias hembras, parte de la varianza de su éxito reproductor puede quedar explicada por las características particulares de cada hembra con la que esté apareado (Veiga *et al.* 2001). Esta estrategia reproductora, no obstante, puede implicar un coste en términos de pérdida de paternidad por la promiscuidad de las hembras (Cordero *et al.* 2003). Como se ha comentado anteriormente, se ha documentado la presencia de pollos extra-pareja en los nidos de estornino negro (10-20% de las nidadas con pollos extra-pareja, Cordero *et al.* 2003), pero no se concluye su relación con el grado de poliginia de los machos. Además las colonias albergan una proporción nada despreciable de individuos flotantes aparentemente no reproductores (Veiga *et al.* datos no publicados). Conocer si estos individuos ganan paternidad a través de conductas promiscuas en el caso de los machos, o si las hembras llegan a ser parásitos de nido intra-específicos como estrategia alternativa de reproducción son algunas de las cuestiones a abordar en esta tesis. La existencia de parásitos intra-específicos entre los pollos de estornino negro ha sido frecuentemente asumida o intuida sobre la base de caracteres morfológicos de los huevos (Calvo *et al.* 2000) o en incongruencias en la secuencia de puesta de los mismos (Veiga *et al.*

datos no publicados), pero si estos huevos llegan o no a dar lugar a embriones viables es una cuestión que permanece aún sin resolver.

Foto 7: Área de estudio

Los datos de campo para la realización de esta tesis fueron recogidos en una colonia de estornino negro establecida a partir de la instalación de 40 cajas nidos en 1996, aunque dada la secuencia de ocupación y la densidad de individuos flotantes observada a lo largo de los años el número de cajas nido instaladas fue aumentado ligeramente. La colonia se sitúa en una finca de 1.3 ha en la falda sur de la sierra del Guadarrama, en el término municipal de Villalba, Madrid (España) a 950 m.s.n.m. Se trata de un paisaje de bosque adehesado de fresnos (*Fraxinus angustifolius*) con quercíneas, mayoritariamente encina (*Quercus rotundifolia*) y roble (*Quercus pyrenaica*) rodeado de terrenos ganaderos adehesados.

2. Marcadores moleculares para el estudio de los patrones de paternidad

El estudio de las relaciones genéticas entre los individuos ha estado tradicionalmente limitado a poblaciones en cautividad o en condiciones controladas en las que es posible tener datos de genealogías completas y por tanto calcular coeficientes de consanguinidad (ejemplo Van Noordwijk y Scharloo 1981; Gibbs y Grant 1989; Thornhill 1993; Keller *et al.* 1994; Cassinello *et al.* 2001). No obstante, la necesidad de ampliar este tipo de estudios a las poblaciones naturales y de una mayor precisión en el establecimiento de las relaciones genéticas ha impulsado durante las últimas décadas el desarrollo de diferentes marcadores genéticos moleculares. Así, tras los primeros estudios de parentesco en poblaciones naturales basados en polimorfismos cromosómicos (Anderson 1974; Milkman y Zeitler 1974; Levine *et al.* 1980), electroforesis de alozimas (Hanken y Sherman 1981; Ellstrand 1984; Meagher 1986) y DNA multilocus-fingerprinting (Burke y Brufort, 1987; Wetton *et al.* 1987), se optimizó el genotipado de loci microsatélites mediante PCR (Litt y Luty 1989; Tautz 1989; Weber y May 1989). Este método es en la actualidad el más ampliamente usado para el diagnóstico de paternidad, la identificación individual y el establecimiento de relaciones de parentesco entre los individuos.

Las razones del éxito de los microsatélites-marcadores genéticos constituidos por secuencias de entre dos y cinco bases de ADN que se repiten en tandem de manera consecutiva normalmente hasta 40 veces (Selkoe y Toonen 2006)-se basan fundamentalmente en que se ajustan en gran medida a la idea de marcador genético ideal, ya que los microsatélites son marcadores muy abundantes en el genoma eucariótico, muy polimórficos (Jarne y Lagoda 1996; Selkoe y Toonen, 2006; Sunnucks 2000; Van Oosterhout *et al.* 2004) y que una vez optimizados permiten reconocer individuos homocigotos y heterocigotos, ya que son codominantes, selectivamente neutros (Schlotterer 2000; Selkoe y Toonen 2006) y presentan segregación mendeliana. Además cada marcador de locus y cada alelo dentro de cada locus es independiente (excepto en los casos raros de ligamiento), por lo que combinar los datos de distintos loci da información más precisa que ayuda a la identificación individual y a reducir los errores de muestreo (Selkoe y Toonen 2006; Sunnucks 2000). A estas razones se unen también ventajas de carácter metodológico, ya que estos marcadores pueden ser analizados mediante PCR y, por tanto, para amplificarlos con éxito no es necesario contar con grandes muestras de tejido para extraer el ADN, ya que al tratarse de secuencias cortas, pueden amplificarse incluso cuando existe cierta degradación del ADN (Queller *et al.* 1993; Jarne y Lagoda 1996; Sunnucks 2000; McPherson y Möller 2000; Selkoe y Toonen 2006). Por otro lado, gracias a los avances biotecnológicos de aparataje y software apropiados, se puede estimar el tamaño de los alelos de cada locus analizado en un individuo con gran precisión y comparar éste con el tamaño de los alelos para ese mismo locus de otros individuos, lo que permite obtener perfiles genéticos de los individuos muy precisos.

3. Manipulaciones hormonales para el estudio de los patrones de paternidad

Las comportamientos promiscuos pueden tener una base fisiológica; así por ejemplo, se ha visto en numerosas especies que el nivel de testosterona es más alto en las especies poligínicas que en las especies monógamas durante la época de cría, lo cual parece estar relacionado con la atracción de un mayor número de hembras (Beletsky *et al.*, 1995). Se han hecho numerosos estudios acerca de los efectos de diferentes niveles de testosterona en machos, que han demostrado sus efecto directo en comportamientos sexuales y reproductores tales como disminución del cuidado parental, aumento de la agresividad, desarrollo de caracteres secundarios o aumento de las EPCs (revisado en Wingfield *et al.* 1990; Schlinger *et al.* 2001; Roberts *et al.* 2004; Hirschenhauser y Oliveira 2006). Las hembras también presentan ciertos niveles de testosterona y su influencia en la fisiología, reproducción y otras conductas, entre ellas algunas potencialmente relacionadas con la defensa y adquisición de parejas, han sido ya mostradas en algunos estudios (revisado en Ketterson *et al.* 2005). No obstante, queda aún por averiguar si la testosterona de una hembra podría estar implicada en su decisión para buscar EPCs. Esto no sería raro, dado que las hembras parecen ejercer un papel activo en la conducta promiscua y que el papel de esta hormona en dicha conducta en el caso de los machos ha sido ya comprobado en algunas especies (Raouf *et al.* 1997)

El modo más sencillo de analizar la posible relación entre la concentración de una hormona y sus posibles efectos es incrementar la concentración de la hormona en los individuos mediante implantes subcutáneos que contengan una dosis no farmacológica. Esta técnica se ha utilizado ya en numerosas ocasiones demostrando su idoneidad (Nolan y Ketterson 1992; Raouf *et al.* 1997; Veiga *et al.* 2001, 2002; Cordero *et al.* 2003; Veiga y Polo 2008; O´Neal *et al.* 2008; López-Rul y Gil 2009).

BIBLIOGRAFÍA

Anderson, W.W. 1974. Frequent multiple insemination in a natural population of *Drosophila pseudoobscura*. *American Naturalist*, **108**: 709–711.

Aparicio, J. M., Cordero, P. J. & Veiga, J. P. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. *Animal Behaviour*, **62**: 1001-1006.

Beletsky, L. D., Gori, D. F., Freeman, S. & Wingfield, J. C.1995 Testosterone and polygyny in birds. In: *Current ornithology*, 12 (Ed. by D. M. Power), pp. 1-41. New York: Plenum Press.

Birkhead, T. R., Hatchwell, B. J. & Lindner, R. 2001. Extra-pair paternity in the common murre. *The Cooper Ornithological Society*, **103**: 158–162.

Bulmer, E., Celis, P. & Gil, D. 2008. Parent-absent begging: evidence for sibling honesty and cooperation in the spotless starling (*Sturnus unicolor*). *Behavioral Ecology*, **19**: 279-284.

Burke, T. & Bruford, M.W. 1987. DNA fingerprinting in birds. *Nature*, **327**, 149-142.

Calvo, J. M., Pascual, J. A., Deceuninck, B. & Peris, S. J. 2000. Intraspecific nest parasitism in the spotless starling *Sturnus unicolor*. *Bird Study*, **47**: 285-294.

Cassinello, J., Gomendio, M. & Roldan, E. R. S. 2001. Relationship between coefficient of inbreeding and parasite burden in endagered gazelles. *Conservation Biology*. **15**: 1171-1174.

Cerit, H. & Avanus, K. 2007. Sex identification in avian species using DNA typing methods. *World's Poultry Science Journal*, **63**: 91-100.

Cordero, P. J., Viñuela, J., Aparicio, J. M. & Veiga, J. P. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *Journal of Evolutionary Biology*, **14**: 829-834.

Cordero P. J., Veiga, J. P., Moreno, J. & Parkin, D. T. 2003. Extra-pair paternity in the nest of facultative polugynous starling *Sturnus unicolor*. *Behavioural Ecology and Sociobiology*, **54**:1-6.

Cramp, S., Perrins, C. M. 1994. *Handbook of the birds of Europe the Middle East and North Africa. Vol VIII.* (Ed. by S. Cramp). Oxford: Oxford University Press.

Cuervo, J. J. & Møller, A. P. 1999. Ecology and evolution of extravagant feather ornaments. *Journal of Evolutionary Biology*, **12**: 986-998.

Eens, M. & Pinxten, R. 1999. Responses of male Spotless Starlings *Sturnus unicolor* to experimental intraspecific nest parasitism vary in relation to the nesting stage. *Journal of Avian Biology*, **30**: 122–125.

Ellstrand, N. C. 1984. Multiple paternity within the fruits of the wild radish, *Raphanus sativus*. *American Naturalist*, **123**: 819–828.

Ferrer, X., Motis, A., & Peris, S. J. 1991. Changes in the breeding range of starlings in the lberian Peninsula during the last 30 years: competition as a limiting factor. *Journal of Biogeography*, **18**: 631-636.

Fridolfsson, A. K. & Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**: 116-121.

Gibbs, H. L. & Grant, P. R. 1989. Inbreeding in Darwin medium ground finches (*Geospiza fortis*). *Evolution,* **43**: 1273-1284.

Gil, D., Bulmer, E., Celis, P. & Lopez-Rull, I. 2008. Adaptive developmental plasticity in growing nestlings: sibling competition induces differential gape growth. *Proceedings of the Royal Society of London, Series B,* **275**: 549-554.

Griffiths, R., Tiwari, B. & Becher, S. A. 1992 The identification of sex in the starling *Sturnus vulgaris* using a molecular DNA technique. *Molecular Ecology*, **1**: 191–194.

Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. 1998. A DNA test to sex most birds. *Molecular Ecology,* **7**: 1071-1075.

Hanken, J. & Sherman, P. W. 1981. Multiple paternity in Belding's ground squirrel litters. *Science*, **212**: 351–353.

Hiraldo, F. & Herrera, C. M. 1974. Dimorfismo sexual y diferenciación de edades en *Sturnus unicolor* Temm. *Doñana Acta Vertebrata*, **1**: 149-170.

Hirschenhauser, K. & Oliveira, R. F. 2006. Social modulation of androgen levels in vertebrates: A meta-analysis of the Challenge Hypothesis. *Animal Behaviour*, **71**: 265-277.

Jarne, P. & Lagoda, P. J. L. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution*, **11**: 424-429.

Jensen, T., Pernasetti, F. M. & Durrant, B. 2003. Conditions for rapid sex determination in 47 avian species by PCR of genomic DNA from blood, shell-memb-rane blood vessels and feathers. *Zoo Biology*, **22**: 561–567.

Kahn, N. W., St. John, J., Quinn, T. W. 1998. Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. *Auk*, **115**: 1074-1078.

Keller, L. F., Arcese, P., Smith, J. N. M., Hochachka, W. M. & Stearns, S. C. 1994. Selection againts inbred song sparrows during a natural population bottleneck. *Nature*, **372**: 356-357.

Ketterson, E. D., Nolan, V. Jr. & Sandell, M. 2005. Testosterone in females: mediator of adaptative traits, constraint on sexual dimorphism, or both?. *American Naturalist*, **166**: 85-98.

Levine, L., Asmussen, M., Olvera, O., Powel, J. R., De la Rosa, M. E., Salceda, V. M., Gaso, M. I., Guzman, J. & Anderson, W. W. 1980. Population genetics of Mexican *Drosophila*. V. A High rate of multiple insemination in a natural population of *Drosophila pseudoobscura*. *American Naturalist,* **116**: 493–503.

Lezana, L., Miranda, R., Campos, F. & Peris, S. J. 2000. Sex differentiation in the spotless starling (*Sturnus unicolor*, Temminck 1820). *Belgian Journal of Zoology,* **130**: 139-142.

Litt, M. & Luty, J.A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics*, **44**: 397-401.

López-Rull, I. & Gil. D. 2009. Elevated testosterone levels affect female breeding success and yolk deposition in a passerine bird. *Behavioural Processes*, **82**: 312-318.

López-Rull, I., Miksik, I. & Gil, D. 2008. Egg pigmentation reflects female and egg quality in the Spotless Starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology,* **62**: 1877–1884.

López-Rull, I., Celis, P., Salaberria, C., Puerta, M. & Gil, D. 2011. Post-fledging recruitment in relation to nestling plasma testosterone and immunocompetence in the spotless starling. *Functional Ecology*, **25**: 500-508.

Martín, A. & Lorenzo J. A. 2001. *Aves del archipiélago canario*. La Laguna: Francisco Lemus.

McPherson, M. J., Möller, A. P. 2000. *PCR*. Oxford: BIOS Scientific Publishers.

Meagher, T. R. 1986. Analyses of paternity within a natural population of Chamaelirium luteum. Identification of most-likely male parents. American Naturalist, 128:199-215.

Milkman, R. & Zeitler, R. R. 1974. Concurrent multiple paternity in natural and laboratory populations of *Drosophila melanogaster*. *Genetics*, **78**: 1191-1193.

Moreno, J., Veiga, J. P., Cordero, P., Mínguez, E. 1999. Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **47**: 47-53.

Müller, W., Deptuch, K., López-Rul, I. & Gil, D. 2007. Elevated yolk androgen levels benefit offspring development in a between-clutch context. *Behavioral Ecology*, **18**: 929-936.

Nolan, V. & Ketterson, E.D. 1992. Testosterone an avian life histories: Effects of experimentally elevated testosterone on prebasic molt and survival in dark-eyed juncos. *The Condor*, **94**: 364-370.

O'Neal, D. M., Reichard, D. G., Pavilis, K. & Ketterson E. D. 2008. Experimentally elevated testosterone, female parental care, and reproductive success in a songbird, the dark-eyed junco (*Junco hyemalis*). *Hormones and Behaviour*, **54**: 571-578.

Peris, S. J. 1989. Biometría del estornino negro *(Sturnus unicolor* Temm.) En el centro-oeste de la Península Ibérica. *Miscelanea Zoologica*, **13**: 217-220.

Pinxten, R., Eens, M. & Verheyen, R. F. 1989. Polygyny in the European starling. *Behaviour*, **111**: 234-256.

Polo, V. & Veiga, J. P. 2006. Nest ornamentation by female spotless starlings in response to a male display: an experimental study. *Journal of Animal Ecology*, **75**: 942-947.

Queller, D. C., Strassmann, J. E. & Hughes, C. R. 1993. Microsatellites and Kinship. *Trends in Ecology & Evolution*, **8**: 285-288.

Raouf, S. A., Parker, P. G., Ketterson, E. D., Nolan, V. & Ziegenfus, C. 1997. Testosterone affects reproductive success by influencing extrapair fertilization in male dark-eyed juncos. *Proceedings of the Royal Society of London, Series B*, **264**: 1599-1603.

Roberts, M. L., Buchanan, K. L. & Evans, M. R. 2004. Testing the immunocompetence handicap hypothesis. *Animal Behaviour*, **68**: 227-239.

Sandell, M. I. & Smith, H. G. 1996. Already mated females constrain male mating success in the European starling. *Proceedings of the Royal Society of London, Series B*, **263**: 743-747.

Schlinger, B. A., Soma, K. K. & Saldanha, C. 2001. Perspectives in ornithology: advances in avian behavioral endocrinology. *Auk*, **118**: 283-289.

Schlotterer, C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*, **109**: 365-371.

Selkoe, K. A. & Toonen, R. J. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9**: 615-629.

Sunnucks, P. 2000 Efficient genetic markers for population biology. *Trends in Ecology & Evolution*, **15**: 199-203.

Tautz, D. 1989. Hypervariability of simple sequences as a general source of polymorphic DNA markers. *Nucleic Acids Research*, **17**: 6463-6471.

Thornhill, N. W. 1993. *The Natural History of Inbreeding and Outbreeding*. Chicago: University of Chicago Press.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley, P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**: 535-538.

Van Noordwijk, A. J. & Scharloo, W. 1981. Inbreeding in an island population of the great tit. *Evolution,* **35**: 674-688.

Veiga, J. P. & Polo, V. 2003. Estornino Negro – *Sturnus unicolor*. In: *Enciclopedia Virtual de los Vertebrados Españoles*. (Ed. by L. M. Carrascal and A. Salvador). Madrid: Museo Nacional de Ciencias Naturales. http://www.vertebradosibericos.org/

Veiga, J. P. & Polo, V. 2008. Fitness consequences of increased testosterona levels in females spotless starling. *American Naturalist*, **172**: 42-53.

Veiga J. P., Moreno, J., Cordero, P. J. & Mínguez, E. 2001. Territory size and polygyny in the spotless starling: resource holding potential or social inertia?. *Canadian Journal of Zoology*, **79**: 1951-1956.

Veiga, J. P., Moreno, J., Arenas, M., Sánchez, S. 2002. Reproductive consequences for males of paternal vs. territorial strategies in the polygynous spotless starling under variable ecological and social conditions. *Behaviour,* **139**: 677-693.

Veiga, J. P., Viñuela, J., Cordero, P. J., Aparicio, J. M., Polo, V. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. *Hormones and Behavior*, **46**: 47-53.

Weber, J. L. & May, P. E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics*, **44**: 388-396

Wetton, J. H, Carter, R. E, Parkin, T. & Walters, D. 1987. Demographic study of a wild House Sparrow population by DNA fingerprinting. *Nature,* **327**: 147-149.

Wingfield, J. C., Hegner, R. E. Jr, Dufty, A. M. Jr. & Ball, G. F. 1990. The `challenge hypothesis': theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136**: 829-846.

Wittenberger, J. F. 1976. The ecological factor selecting for polygyny in altricial birds. *American Naturalist*, **110**: 779-799.

OBJETIVOS

El objetivo general de esta tesis es tratar de entender la conducta promiscua del estornino negro (medida principalmente como presencia de pollos extra-pareja (EPOs) en sus nidos) desde una doble perspectiva, funcional y evolutiva, y analizar cómo influyen ambos sexos y sus características en la elección de esta estrategia en el marco de un conflicto sexual en el que las decisiones de cada uno de los sexos están directamente influidas por las del otro (Carranza 1994). Además, con la manipulación experimental de la fisiología hormonal de las hembras, intentaremos analizar los efectos de la variación de las estrategias de reproducción y sus repercusiones en el éxito reproductor de los individuos. Para ello se contemplarán como objetivos particulares los siguientes:

1) Obtener y optimizar un panel de marcadores para microsatélites polimórficos de amplificación cruzada válidos para su empleo en estudios genéticos en el estornino negro y, en concreto, en estudios de paternidad, paternidad extra-pareja, pseudoparasitismo y parasitismo intra-específico. (Capítulo 1)

2) Investigar si el estatus hormonal de las hembras, y en concreto sus niveles de testosterona, influyen en el control que éstas pueden ejercer sobre las cópulas extra-pareja (EPCs). ¿Puede la testosterona plasmática influir en el estatus de emparejamiento de las hembras y en su decisión de buscar machos extra-pareja? ¿Son los efectos no deseados de la testosterona en hembras los responsables de que estas se reproduzcan bajo un sistema poligínico que es ventajoso para los machos pero perjudicial para ellas? (Capítulo 2)

3) Investigar si existen variaciones en el cuidado parental de los machos en función de su confianza de paternidad. Dado que las fertilizaciones extra-pareja son frecuentes en el estornino negro, un aspecto importante a dilucidar es si los machos que tienen EPOs en su nido ceban menos que los machos que no sufren pérdida de paternidad. Este análisis es interesante, puesto que la reducción en el cuidado parental ejercido por los machos puede representar uno de los mayores costes de la paternidad extra-pareja para las hembras de muchas especies de aves, cuando el

control de las cópulas extra-pareja recae en ellas. Desde una perspectiva evolutiva, este análisis arrojaría nuevos datos acerca de las posibles causas del mantenimiento de la paternidad extra-pareja como estrategia reproductora. (Capítulo 3)

4) Evaluar la importancia relativa que los atributos fenotípicos (condición física, experiencia y ornamentos) tanto de los machos como de las hembras tienen en los niveles de paternidad extra-pareja en el estornino negro. Si las conductas promiscuas están relacionadas con la búsqueda de individuos de mayor calidad que la pareja social y aceptamos el fenotipo como un reflejo de la calidad de los mismos, esperaríamos encontrar diferencias fenotípicas entre los individuos que participasen activamente en este tipo de estrategias y los que no lo hicieran o tuvieran, a su vez, EPOs en sus nidos (Capítulo 4)

5) Abordar desde una aproximación multihipótesis (hipótesis de la diversidad genética, hipótesis de garantía de la fertilidad, hipótesis de los "buenos genes" e hipótesis de compatibilidad genética) las posibles causas de la elección de la paternidad extra-pareja como estrategia reproductora por parte de las hembras. Este estudio complementa al anterior, ya que no se centra tan sólo en las diferencias entre los individuos adultos, sino también en las diferencias entre los pollos producidos a través de EPCs y aquellos otros que son hijos genéticos de la pareja social (Capítulo 5).

CAPÍTULO 1

Microsatélites polimórficos de amplificación cruzada para el estornino negro (*Sturnus unicolor)*

Elena García-Vigón, Pedro J. Cordero & José Pablo Veiga. 2008.
Cross-amplified polymorphic microsatellites for the Spotless
Starling *Sturnus unicolor*, *Ardeola*, **55**: 3-11.

RESUMEN

Objetivos: Contrastar la validez real de 18 cebadores para microsatélites de amplificación cruzada para el genotipado de individuos de estornino negro *Sturnus unicolor* y, por tanto, expandir el panel de microsatélites realmente útiles para realizar estudios genéticos en esta especie.

Localidad: Villalba, Madrid, Spain.

Métodos: Se usaron como punto de partida 18 cebadores para microsatélites aislados a partir de 7 especies distintas de aves. Su reacción en cadena de la polimerasa (PCR) fue optimizada. Se analizó el polimorfismo y la variabilidad genética de aquellos cebadores que producían bandas del tamaño esperado y tenían un electroferograma y patrón de segregación claro. Para testar la verdadera utilidad de estos cebadores en estudios genéticos, se decidió usarlos para analizar la frecuencia de paternidad extra-pareja (EPP), parasitismo intra-específico (IBP) y pseudo-parasitismo en 30 parejas y 206 pollos.

Resultados: De los 18 microsatélites originales, se seleccionaron 6 por su polimorfismo (promedio 8 alelos por locus), heterocigosis (heterocigosis esperada: 0.656) y claro patrón de segregación. Se detectaron casos de EPP (16.5 % de los pollos y el 51 % de las puestas) y pseudo-parasitismo (0.97% de los pollos y 3.5 % de las puestas). No se detectó ningún caso de IBP.

Conclusiones: Este trabajo proporciona 6 marcadores polimórficos de amplificación cruzada en el estornino negro y testa su validez para estudios genéticos en esta especie en un número razonable de individuos, confirmado así, su utilidad en posibles estudios futuros de diversidad genética, estructura de la población, identificación de individuos y estrategias reproductoras.

Palabras clave: microsatélites de amplificación cruzada, paternidad extra-pareja, pseudo-parasitismo, *Sturnus unicolor*.

Cross-amplified polymorphic microsatellites for the spotless starling *Sturnus unicolor*

ABSTRACT

Aims: To test the real suitability of a set of 18 cross-amplified microsatellite primers for individual genotyping of the spotless starling and, therefore, to expand the panel of available microsatellite really useful for genetic studies of this species.

Location: Villalba, Madrid, Spain.

Methods: 18 microsatellite primers isolated from 7 different bird species were used as starting point. Their Polymerase Chain Reaction (PCR) was optimized. The polymorphism and genetic variability of primers that produced bands of expected size and had a clear electropherogram and segregation pattern was checked. To test the real suitability of these primers in genetic studies, they were used to analyse the frequency of extra-pair paternity (EPP), intra-specific brood parasitism (IBP) and pseudo-parasitism in 30 breeding pairs and 206 offspring.

Results: Six of the 18 initial primers were selected by their polymorphism (average alleles per locus: 8), heterozygosity (expected heterozygosity: 0.656), segregation and unambiguous electropherogram pattern. EPP was 16.5 % of offspring and 51 % of clutches. Pseudo-parasitism was 0.97 % of offspring and 3.5 % of clutches. IBP was not detected.

Conclusions: This paper provides 6 cross-amplified polymorphic markers for spotless starling and tests their suitability for genetic studies in this species in a reasonable number of individuals, confirming their utility in possible future studies of genetic diversity, population structure, individual identification and reproductive strategies.

Keywords: cross-amplified microsatellites, extra-pair paternity, pseudo-parasitism, *Sturnus unicolor*.

INTRODUCTION

The spotless starling *Sturnus unicolor* is a facultatively polygynous passerine living in large permanent social groups which is being intensively studied (Moreno *et al.* 1999; Veiga *et al.* 2001, 2002, 2004; Polo *et al.* 2004; Polo & Veiga, 2006). Their laying period extends from mid April to mid June and most females lay two clutches per breading season. Both males and females defend nest boxes against competitors and intra-sexual competition for nest sites is heavy in both males and females. Breeding is mostly synchronous and alternative breeding strategies such as promiscuity and intra-specific brood parasitism (IBP) had been detected using DNA multilocus fingerprinting (Cordero *et al.* 2003). Also by using DNA multilocus fingerprinting Aparicio *et al.* (2001) provided a new index for individual homozygosity based on the frequency of bands shared between parents and offspring used for studies on heterozygosity fitness correlations (Aparicio *et al.* 2001; Cordero *et al.* 2004). However, the use of this type of DNA marker and others like RAPDs, and single-locus minisatellites can have significant methodological or logistic disadvantages, as they are more time consuming and provide less accurate information on alleles segregation limiting the utility of their application to a particular population study (Dawson *et al.* 1997). Microsatellites are polymorphic markers PCR-based requiring minimal amounts of DNA and therefore less invasive methods for sampling. They are relatively cheap, their analysis is faster than for traditional genetic markers and they present locus-specificity and co-dominance. The advantages of the use of microsatellites as genetic markers seem to be evident, and their characteristics match with those described by Quellar *et al.* (1993) for the ideal genetic marker. Furthermore, microsatellite DNA is relatively abundant in the genomes of many taxa and loci have been isolated from a variety of wild vertebrates (Amos *et al.* 1992; Taylor *et al.* 1994; Paetkau & Strobeck, 1994) including avian species (Ellegren 1992; Hanotte *et al.* 1994; McDonald & Potts 1994; Primmer *et al.* 1995, 1996; Gibbs *et al.* 1996). One of the drawbacks for the use of microsatellites is that they require a complete and time-consuming process for isolation from the focal study species. Recently, nine specific microsatellites have been developed and tested for efficiency in the spotless starling (Celis *et al.* 2007). However, increasing the panel of amplifiable microsatellites is beneficial for the analyses of individual heterozygosity and pair genetic similarity. An alternative to specific microsatellites isolation is testing the cross-amplification of microsatellites already available from different taxa (Galbusera *et al.* 2000). This is a cheaper and faster method to obtain amplifiable microsatellites for a study species and it has been used for a number of avian species (Arruga *et al.* 1996; Dallimer 1999; Dawson *et al.* 2000; Galbusera *et al.* 2000; Loyau *et al.* 2005; Primmer *et al.* 2005). In this paper 18 cross-amplified microsatellites are tested for their suitability in individual genotyping at the spotless starling to try to expand the panel of available microsatellites useful for this species, in which there are still many evolutionary questions to be solved on genetic diversity and fitness, sexual selection and mating strategies.

MATERIAL AND METHODS

DNA extraction

Blood samples were obtained from adults when they were captured and from the nestlings when they were about 5-6 days old, by puncturing the jugular vein. About 0.1 ml of blood was stored in the field in absolute ethanol and then kept at -40 ºC. DNA was extracted and purified from blood samples using NucleoSpin ® Tissue Kit (Macherey-Nagel).

Primers´s amplification

Seven primers isolated from *Acrocephalus sechellensis*, *Aphelocoma ultramarina*, *Catharus ustulatus*, *Fycedula hypoleuca*, *Parus caeruleus and Poecille atricapillus* and 11 primers initially developed for *Lamprotornis superbus* and tested for amplification, but not polymorphism, in the spotless starling (Rubenstein 2005) were used to test for their cross-amplification in spotless starling. (Table 1, Appendix 1). Polymerase chain reaction (PCR) was optimized and carried out on a Mastercycler Epgradients (Eppendorf) thermal cycler using a 40-60 ºC annealing temperature gradient. Approximately 5-10 ng of template DNA were added to 15 µl reaction volumes containing 1X Biotools standard buffer (75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, Biotools), 0.2 mM of each dNTP, 0.2 µM of each primer and 0.5 U of Taq DNA polymerase (Biotools). The PCR programme used was 4 min denaturing at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature and 45 s at 72 °C, ending with 5 min elongation stage at 72 °C. PCR products were visualized under UV light after electrophoresis on a 2.5 % agarose gel stained with ethidium bromide.

Primers's selection and individual genotyping

Primers producing bands of expected size were labelled with fluorescent dyes (FAM, HEX or NED) at the 5′end and checked for polymorphism in 70 unrelated individuals of the total sample studied. Approximately 10 ng of template DNA were amplified in 13 µl reaction volumes containing Ecostart® Reaction Buffer 1X((16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl (Ph 8.3 a 25 °C); 0.01% Tween-20), 0.2 mM dNTPs, 0.25 µmol of each labelled primer and 0.02 U of Taq DNA polymerase (Ecostart®, Ecogen). Polymerase chain reaction (PCR) was run on a GeneAmp® PCR System 9700 (Applied Biosystem) thermal cycler following a denaturation step at 95 °C for 7 min followed by 40 cycles of 30 s at 94 °C; 45 s at the annealing temperature and 45 s at 72 °C, ending with a 5 min elongation stage at 72 °C. Finally, 5 µl of the PCR product was run on a 2.5 % agarose gel to verify amplification. PCR products were sized using a ROX labelled size standard in a single-capillary automated DNA sequencer (ABI 310® Genetic Analyzer, Applied Biosystems) and genotypes were scored using GeneScan 3.7 (Applied Biosystems). In cases where product signal was too low for accurate allele sizing, PCR on the sample was repeated. The primers which have a clear electropherogram pattern were selected and their variability was analysed using CERVUS 2.0 (Marshall *et al.* 1998), GENEPOP 3.4 (Raymond & Rousset 1995) and CERNICALÍN (Aparicio *et al.* 2006).

Parentage analysis

The selected primers were used to analyse the frequency of EPP, IBP and pseudo-parasitism in a breeding colony of Villalba (Madrid, Spain; Moreno *et al.* 1999) during four breeding seasons. The genetic relationships of 206 nestlings, 20 breeding females and 27 putative social males not submitted to any hormonal manipulation experiment (Veiga *et al.* 2004) was determined by comparing nestling genotypes firstly to that of the attending female and then to the territorial male at the six loci. Within-pair young was considered if nestlings alleles matched (*i.e.* if they possessed alleles of the size of each parent and segregation was compatible with Mendelian inheritance) to the social parents alleles on all six loci or had one mismatched allele assuming that this mismatch could be originated through mutation. Nestlings that mismatched the social male allele at two or more loci were considered to be extra-pair offspring (Johnsen *et al.* 1998). Those that mismatched the social female allele at two or more loci but matched the social male were considered as pseudo-parasite offspring. Nestlings that mismatched both social male and female alleles at two or more loci were considered to be intra-specific-parasite offspring. The offspring (if the other siblings matched) or the parents (if all siblings mismatched in two or more loci) were genotyped again for all parent-offspring relationships with two or more mismatches.

RESULTS AND DISCUSSION

Identification and characterization of microsatellite loci useful for genetic analyses

Of the 18 initial primers 15 were polymorphic in spotless starling and of these 6 were selected for their unambiguous and clear electropherogram and their segregation pattern. (Table 1, Appendix 1). These loci produced 48 alleles and were polymorphic with an average of 8 alleles per locus (range: 4- 12). Average proportion of heterozygotes was 0.669. The frequency of expected (0.656; range: 0.431- 0.815) and observed (0.682; range: 0.457- 0.841) heterozygosity did not differ (X^2_{12} = 4.1; P = 0.9822) indicating no departure from

Hardy-Weinberg equilibrium and an absence of a significant proportion of null alleles (*sensu* Pemberton *et al.* 1995; Primmer *et al.* 1995). All tests for pair-wise linkage disequilibrium between loci were non-significant. The probability of parental exclusion at each locus ranged between 0.269 and 0.651 and the combined probability of parental exclusion for all six loci together was 0.977.

Use of microsatellite markers for parentage analysis

The real suitability of the 6 selected microsatellites for genetic studies was assessed using them to analyse extra-pair paternity, pseudo-parasitism and intraspecific-brood parasitism in the study population. The 16.5% (34/206) of chicks resulted from extra-pair fertilization and 51% of clutches (29/57) contained at least one extra-pair young. Pseudo-parasitism occurred in 0.97% (2/206) of chicks from 3.5% of clutches (2/57) but we did not detect IBP, results congruent with previous work (Cordero *et al.* 2003).

In sum, the primary result of this paper is that it provides a set 6 cross-amplified microsatellites for spotless starling that have been already tested in a full population (253 individuals) showing that they are efficient for studies of parentage and could be also used for studies of genetic diversity, dispersal, population substructure and genetic correlates of mating and breeding success in this species

Table 1. –Characterization of 6 selected cross-amplified microsatellite loci for spotless starling (*Sturnus unicolor*). Primer name, initial species, original locus source reference, GenBanK Accession numbers, primer sequences, primer-dye, annealing temperatures (Ta), number of individuals tested, number of alleles, size range, observed heterozygosity (HO), expected heterozygosity (HE), exact *P*-values and SE of the Hardy-Weinberg equilibrium test for six cross-amplified microsatellite loci selected in spotless starling.

Locus	Initial species	Ref	GenBank Accession no.	Primer sequences (5´-3´)	Primer-Dye	T_a(°C)	No. Of indiv.	No. Of alleles	Size range (bp)	Ho	He	HW p-value	HW SE
Pca-7	*Parus caeruleus*	*Dawson et al., 2000*	AJ279809	F- TGA GCA TCG TAG CCC AGC AG R- GGT TCA GGA CAC CTG CAC AAT G	HEX	56	70	12	92-128	0.457	0,431	0.8518	0.0206
Ase-18	*Acrocephalus sechellensis*	Richardson et al. 2000	AJ276375	F- ATC CAG TCT TCG CAA AAG CC R- TGC CCC AGA GGG AAG AAG	NED	50	70	7	176-198	0.771	0,8	0.7504	0.0048
SS 2 32	*Lamprotornis superbus*	Rubenstein. 2005	AY906889	F- GGT ATC ACC ATA TCT GCT GCC AGT A R- CAG GCT TTT GCT GAC AAT TAT TTT G	FAM	58	69	12	228-254	0.841	0,815	0.7798	0.0098
SS 1 6	*Lamprotornis superbus*	Rubenstein. 2005	AY906875	F- TTT CAC TGG CTG GAT CTG GTA AAC C R- CTA GCA ACA TAT AGC CCA AGC TGT ATT GAT	HEX	60	70	8	188-204	0.814	0,757	0.4113	0.0091
SS 2 106	*Lamprotornis superbus*	Rubenstein. 2005	AY906877	F- TGT GTT ATC CCA TTG TAA GGG CTC TTT R- GAC TCT AGG TGG AAA CCC CCA TTT T	FAM	56	70	4	272-284	0.514	0.525	0.8698	/
SS 2 130	*Lamprotornis superbus*	Rubenstein. 2005	AY906884	F- CTG AAG GCA CCC AGC AGG TTC T R- AGA CCC ACT GTG ATA ATT ACC ACT TCT CTG	NED	58	70	5	242-252	0.700	0,608	0.7343	0.0048

ACKNOWLEDGEMENTS

Dustin R. Rubenstein kindly supplied details about amplification conditions of his microsatellites. J. M. Aparicio provided and explained the program CERNICALÍN. J. Ortego made useful suggestions and comments for the laboratory work. E. García-Vigón. was supported by a pre-doctoral fellowship from the Ministerio de Educación y Ciencia (MEC). This work received financial support from MEC projects: CGL-2005-05611-C02-01 y -02 /BOS.

REFERENCES

Amos, B., Schlötterer, C. & Tautz, D. 1992. Social structure of pilot whales revealed by analytical DNA profiling. *Science*, **260**: 670 -672.

Aparicio, J. M., Cordero, P. J. & Veiga, J. P. 2001. A test of hypothesis of mate choice based on heterozygosity in the Spotless Starling. *Animal Behaviour*, **62**: 1001-1006.

Aparicio, J. M., Ortego, J. & Cordero, P. J. 2006. What should we weigh to estimate heterozygosity, alleles or loci?. *Molecular Ecology*, **15**: 4659-4665.

Arruga, M. V., Villarroel, M. R. & Heriz, A. 1996. Amplification of microsatellite DNA loci of Spanish Partridges (*Alectoris rufa* and *A. graeca*) by chicken-specific primers. *Animal Genetics*, **27**: 27.

Celis, P., Gil, D. & Graves, J. A. 2007. Isolation and characterization of polymorphic microsatellites isolated from the Spotless Starling (*Sturnus unicolor*) and cross-species amplification in the European Starling (*Sturnus vulgaris*). *Molecular Ecology Notes*, **7**: 251-253.

Cordero, P. J., Veiga, J. P., Moreno, J. & Parkin, D. T. 2003. Extra-pair paternity in the facultative polygynous Spotless Starling, *Sturnus unicolor*. *Behavioural Ecology and Sociobiology*, **54**: 1-6.

Cordero, P. J., Aparicio, J. M. & Veiga, J. P. 2004. Parental genetic characteristics and hatching success in the Spotless Starling, *Sturnus unicolor*. *Animal Behaviour*, **67**: 637-642.

Dallimer, M. 1999. Cross-species amplification success of avian microsatellites in the Red Billed Quelea *Quelea quelea*. *Molecular Ecology*, **8**: 695-698.

Dawson, D. A., Hanotte, O., Greig, C., Stewart,I. R. K. & Burke, T. 2000. Polymorphic microsatellites in the Blue Tit *Parus caeruleus* and their cross-species utility in 20 songbird families. *Molecular Ecology*, **9**: 1941-1943.

Dawson, R. J. G., Gibbs, H. L., Hobsons, K. A. & Yezerinac, S. M. 1997. Isolation of microsatellite DNA markers from a passerine bird, *Dendroica petechia* (the Yellow Warbler), and their use in population studies. *Heredity*, **79**: 506-514.

Ellegren, H. 1992. Polymerase chain reaction (PCR) analysis of microsatellites- a new approach to the study of genetic relationships in birds. *Auk*, **109**: 886-895.

Galbusera, P., Van Dongen, S. & Matthysen, E. 2000. Cross-species amplification of microsatellite primers in passerine birds. *Conservation Genetics*, **1**: 163-168.

Gibbs, H. L., Brooke, M. De L. & Davies, N. B.1996. Analysis of genetic differentiation of host races of the Common Cuckoo *Cuculus canorus* using mitochondrial and microsatellite DNA variation. *Proocedings of the Royal Society of London, Serie B*, **263**: 89-96.

Hanotte, O., Zanon, C., Pugh, A., Greig, C., Dixon, A. & Burke, T. 1994. Isolation and characterization of microsatellite loci in a passerine bird: the Reed Bunting *Emberiza schoeniclus*. *Molecular Ecology*, **3**: 529-530.

Johnsen, A., Andersson, S., Ornborn, J. & Lifjeld, J.T. 1998. Ultraviolet plumage ornamentation affects social mate choice and sperm competition in Bluethroats. *Proocedings of the Royal Society of London, Serie B*, **265**: 1313-1318.

Li, S-H., Huang,Y-J. & Brown, J. L. 1997. Isolation of tetranucleotide microsatellites from the Mexican Jay *Aphelocoma ultramarina*. *Molecular Ecology*, **6**: 499-501.

Lislegibbs, H., Tobak, L. M. & Hobson, K. 1999. Characterization of microsatellite DNA loci for a neotropical migrant songbird, the Swainson's Thrush (*Catharus ustulatus*). *Molecular Ecology*, **8**: 1551-1552.

Loyau, A., Moureau, B., Richard, M., Christe, P., Heeb, P. & Sorci, G. 2005. Cross-amplification of polymorphic microsatellites reveals extra-pair paternity and brood parasitism in *Sturnus vulgaris*. *Molecular Ecology Notes*, **5**: 135-139.

Marshall, T. C., Slate, J., Kruuk, L. E. B. & Pemberton, J. M.1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **4**: 639-655.

Mcdonald, D. B. & Potts, W. K. 1994. Cooperative display and relatedness among males in a lek-mating bird. *Science*, **266**: 1030-1032.

Moreno, J., Veiga, J. P., Cordero, P. J. & Mínguez, E. 1999. Effects of paternal care on reproductive success in the polygynous Spotless Starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **47**: 47-53.

Otter, K., Ratcliffe, L., Michaud, D. & Boag, P. T. 1998. Do female Black-Capped Chickadees prefer high ranking males as extrapair partners? *Behavioural Ecology and Sociobiology*, **43**: 25-36.

Paetkau, D. & Strobeck, C. 1994. Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **3**: 489-495.

Pemberton, J. M., Slate, J., Bancroft, D. R. & Barret, J. A. 1995. Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**: 249-252.

Polo, V., Veiga, J. P., Cordero, P. J., Viñuela, J. & Monaghan, P. 2004. Female starlings adjust primary sex ratio in response to aromatic plants in the nest. *Proocedings of the Royal Society of London, Serie B*, **271**: 1929-1933.

Polo, V. & Veiga, J. P. 2006. Nest ornamentation by female Spotless Starlings in response to a male display: an experimental study. *Journal of Animal Ecology*, **75**: 942-947.

Primmer, C. R., Møller, A. P. & Ellegren, H. 1995. Resolving genetic relationships with microsatellite markers; a parentage testing system for the swallow *Hirundo rustica*. *Molecular Ecology*, **4**: 493-498

Primmer, C. R., Møller, A. P. & Ellegren, H. 1996. A wide range survey of cross-species microsatellite amplification in birds. *Molecular Ecology*, **5**: 365-378.

Primmer C. R., Painter, J. N., Koskinen, M. T., Palo, J. U. & Merilä, J. 2005. Factors affecting avian cross-species microsatellite amplification. *Journal of Avian Biology*, **36**: 348-360.

Quellar, D. C., Strassmann, J. E. & Hughes, C. R. 1993. Microsatellites and kinship. *Trends in Ecology & Evolution*, **8**: 285-288.

Raymond, M. & F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact test and ecumenicism. *Journal of Heredity*, **86**: 248-249.

Richardson, D. S., Jury, F. L., Dawson, D. A., Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles Warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in *Sylviidae* species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**: 2226-2231.

Rubenstein, D. R. 2005. Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding Superb Starling, *Lamprotornis superbus*. *Molecular Ecology Notes*, **5**: 739-744.

Taylor, A. C., Sherwin, W. B. & Wayne, R. K. 1994. Genetic variation of microsatellite loci in a bottle-necked species: the northern hairy-nosed wombat, *Lasiorhinus krefftii*. *Molecular Ecology*, **3**: 277-290.

Veiga, J. P., Moreno, J., Cordero, P. J. & Mínguez, E. 2001. Territory size and polygyny in the Spotless Starling: resource holding potencial or social inertia?. *Canadian Journal of Zoology*, **79**:1-6.

Veiga, J. P., Moreno, J., Arenas, M. & Sánchez, S. 2002. Reproductive consequences for males paternal vs. territorial strategies in the polygynous Spotless Starling under variable ecological and social conditions. *Behaviour*, **139**: 677-693.

Veiga, J. P., Viñuela, J., Cordero, P. J., Aparicio, J. M.

& Polo, V. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the Spotless Starling. *Hormones and Behaviour*, **46**: 47-53.

APPENDIX 1

Characterization of other microsatellite loci (12) tested for cross amplification in the spotless starling (*Sturnus unicolor*) that were discarded for one or more of the following reasons: poor or no amplification in spotless starling, non-specific amplification products with multiband profiles, non scoreable or detection of only one or two alleles.

Locus	Initial species	Ref.	GenBank Accession no.	Primer sequences (5´-3´)	Ta(°C)	No. of indiv.	Amplification	No. of alleles	Size range (bp)
Ase-19	*Acrocephalus sechellensis*	Richardson et al., 2000	AJ276376	F- TAG GGT CCC AGG GAG GAA G R- TCT GCC CAT TAG GGA AAA GTC	57	14	yes	3	148-172
Cuu-28	*Catharus ustulatus*	Lisle et al., 1999	AF122894	F- GAG GCA CAG AAA TGT GAA TT R- ACT TAT TTC AGT CCT AAA TTC ACC	60	10	yes	2	127-131
Patmp 2.43	*Poecille atricapillus*	Otter et al., 1998	AM056064	F- ACA GGT AGT CAG AAA TGG AAA G R- GTA TCC AGA GTC TTT GCT GAT G	63	20	yes	4	120-134
SS 1.11	*Lamprotornis superbus*	Rubenstein, 2005	AY906870	F- AAA TTT GAA CCG ATC CAG CCT GTT TA R- CTC GCT CCC TCT CCC TCT TTC AC	61	17	yes	5	176-188
SS 1.12	*Lamprotornis superbus*	Rubenstein, 2005	AY906871	F- TAT TTT CCC TTT TCT TCC CTT AGC AG R- GAA GCC AAC AAT ATG TAC AGA ATG TGC	60	7	yes	4	205-213
SS 2.68	*Lamprotornis superbus*	Rubenstein, 2005	AY906905	F- AAC TTG CTG GTT GAA AAT TTT AAT G R- TGT TCT TTA ATT GTT ACT CAG AAG TGA A	60	16	yes	2	134-136
SS 2.80	*Lamprotornis superbus*	Rubenstein, 2005	AY906910	F- ACC CAC TTT TAC CTA CCT AGC ATG TTC TGT R- ATT AGA GTG CCC AAG GAC TTG TTC TCA	58	19	yes	5	304-314
SS 2.83	*Lamprotornis superbus*	Rubenstein, 2005	AY906912	F- TGT CCC AGT TTT ACA TTT TTG TTA A R- GAC TTT TAA TAT GGA GCC TGC TCT	58	8	yes	4	158-164
SS 3.42 C	*Lamprotornis superbus*	Rubenstein, 2005	AY906917	F- TAT ATC CCA GGG AGG GTT GTG GTG TG R- ATC AAA CTG CAG CAG GAC TCT GAC TGT G	60	14	yes	5	142-150
FhU-3	*Ficedula hypoleuca*	Primmer et al., 1996	X84362	F- ATA TTC CCC ATA AGA TAA TGG R- ATA GTG TTG TCT TAA GGT CTC T	49	15	no	–	–
Mjg-1	*Aphelocoma ultramarina*	Li et al., 1997	U82673	F- CCC GGG AAA GGC TTC GTC TTC R- GGA GAT TTT ATA TCG GTG GC	60	10	no	–	–
SS 2.71B	*Lamprotornis superbus*	Rubenstein, 2005	AY906906	F- CAC ACC CAA CAT GTA ACA AAT CTT ACA R- CTT TGA GCC TCT GCT TTT AGA AAT TG	62	11	no	–	–

CAPÍTULO 2

La testosterona exógena en hembras de estornino negro reduce la tasa de pollos extra-pareja

Elena García-Vigón, Pedro J. Cordero & José Pablo Veiga. 2008.
Exogenous testosterone in female spotless starlings reduces their
rate of extra-pair offspring. *Animal Behaviour*, **76**: 345-353

RESUMEN

Los efectos de la testosterona exógena sobre la biología reproductora de las hembras en aves son en gran medida desconocidos. Una cuestión sin solventar aún es si la testosterona tiene un papel en las estrategias de emparejamiento de las hembras, que afecte, por ejemplo, al número de pollos extra-pareja (EPO) obtenidos. En este estudio aumentamos experimentalmente los niveles de testosterona circulantes en las hembras de una colonia libre de estornino negro, *Sturnus unicolor*, y medimos las tasas de paternidad mixta en sus polladas. Las hembras tratadas con testosterona tuvieron un porcentaje significativamente reducido de EPO en relación a las hembras control y esta diferencia persistió en los años siguientes al tratamiento. Además, la edad de las hembras estuvo negativamente relacionada con los EPO, mientras que el número de pollos lo estuvo positivamente. Para nuestro conocimiento, este es el primer estudio sobre hembras en el que los EPO se han analizado en relación a una manipulación experimental de la testosterona. Nosotros sugerimos que las hembras tratadas con testosterona son menos atractivas para los machos que buscan hembras extra-pareja para aparearse y que hay efectos a largo plazo de la testosterona sobre el emparejamiento de las hembras que persisten durante años.

Palabras clave: paternidad extra-pareja, testosterona de la hembra, estrategia reproductora, estornino negro, *Sturnus unicolor*.

Exogenous testosterone in female spotless starlings reduces their rate of extra-pair offspring

ABSTRACT

The effects of exogenous testosterone on the breeding biology of avian females are largely unknown. One unanswered question is whether testosterone has a role in female mating strategies affecting, for example, the rate of extra-pair offspring (EPO) obtained. We experimentally increased circulating testosterone in females of a free-ranging population of the spotless starling, *Sturnus unicolor*, and measured rates of mixed paternity in their broods. Females treated with exogenous testosterone had a significantly reduced percentage of extra-pair offspring in relation to controls and this difference persisted in the years after treatment. In addition, female age was negatively, and the number of nestlings was positively, related to EPO. To our knowledge, this is the first study on females in which EPO has been analysed in relation to an experimental testosterone manipulation. We suggest that females treated with testosterone are less attractive to males seeking extra-pair mates and that there are delayed effects of testosterone on female mating that persist for years.

Keywords: extra-pair paternity, female testosterone, reproductive strategy, spotless starling, *Sturnus unicolor*.

INTRODUCTION

It is well known that testosterone affects mating, breeding and territorial behaviour of males of various vertebrate species (reviewed in Wingfield *et al.* 1990; Schlinger *et al.* 2001; Roberts *et al.* 2004; Hirschenhauser & Oliveira 2006). Both experimental and correlative studies have documented effects of testosterone in controlling sexual and reproductive behaviours particularly in birds, that is, in stimulating territorial aggressiveness and mate attraction tactics (Watson & Parr 1981; Balthazart 1983; Moore 1984; Wingfield 1984; Ketterson

& Nolan 1994, 1999; Enstrom *et al.* 1997; Hunt *et al.* 1997; Wingfield *et al.* 2001), inducing the development of secondary sexual characters (Hillgarth & Wingfield 1997) including male ornamentation (reviewed in Nelson 2000; also Buchanan *et al.* 2001) and enhancing courtship and mating behaviours (McGlothlin *et al.* 2004; Zysling *et al.* 2006) and higher rates of extra-pair fertilizations (EPFs; Raouf *et al.* 1997; see, however, Foerster & Kempenaers 2004). In fact, circulating testosterone levels and rates of extra-pair offspring (EPO) are positively correlated in various bird taxa (Garamszegi *et al.* 2005). High levels of circulating testosterone may also have general repercussions for individual survival (e.g. Dufty 1989; Nolan *et al.* 1992; Reed *et al.* 2006), probably because of its multiple influences in avian physiology (Hannsler & Prinzinger 1979; Feuerbacher & Prinzinger 1981; Wingfield 1984; Ketterson *et al.* 1991, 2001). Increased testosterone may reduce the immune response, increasing the incidence of parasites (Folstad & Karter 1992; Duffy *et al.* 2000; Eens *et al.* 2000; Casto *et al.* 2001; De Ridder *et al.* 2002; Mougeot *et al.* 2004; Owen-Ashley *et al.* 2004; reviewed in Roberts *et al.* 2004). It may also increase the risks of injury (Beletsky *et al.* 1995), reduce male parental care (Schoech *et al.* 1998; Moreno *et al.* 1999) and lower fertility by reducing spermatogenesis (Desjardins & Turek 1977) or ejaculate size (Kast *et al.* 1998).

Females also have certain levels of circulating testosterone, but in this sex the effects are largely unknown (Wingfield 1994; Staub & De Beer 1997; Ketterson *et al.* 2005; Sandell 2007). In females, it is synthesized primarily in the ovaries, by cells in the follicle wall, but also in other tissues, for example the adrenals and perhaps even the brain (Arnold 2004) and it affects normal female functioning. In fact, levels of testosterone in males and females are correlated across species (Ketterson *et al.* 2005; Møller *et al.* 2005) suggesting that the concentration may be controlled by the same genetic factors in both sexes. As in males, testosterone in females is also subject to seasonal fluctuations and an experimental increase in levels in females may cause physiological, morphological and behavioural changes making them more similar to males (Ketterson *et al.* 2005). Administration of testosterone within the physiological range may also affect mating. Higher levels of testosterone enhance female-female aggression (Searcy 1988; Adkins-Regan 1999; Zysling *et al.* 2006; Sandell 2007) and stimulate song (e.g. Kern & King 1972; Arcese *et al.* 1988; Hausberger *et al.* 1995; Nespor *et al.* 1996), traits potentially related to the defence and acquisition of mates and clearly in accordance with those shown by males with experimentally increased testosterone. Testosterone may also make females less selective when choosing males (McGlothlin *et al.* 2004). Because they are more male like, testosterone-treated females may become less attractive to potential mates (Ketterson *et al.* 2005). Another effect of testosterone on females is a delay in laying and a reduction in clutch size (Searcy 1988; Clotfelter *et al.* 2004; Rutkowska *et al.* 2005). When selection strongly favours a testosterone dependent trait in males but the trait may be detrimental to females in some way, a correlated response to selection in females could slow the rate of evolution of this trait in males (McGlothlin *et al.* 2004; Ketterson *et al.* 2005; Møller *et al.* 2005).

Given the pleiotropic effects of testosterone on female birds, which are in part mirrored by those found in males, one unanswered question is whether testosterone affects rates of EPFs and, in natural populations, changes mate selection by females. We investigated this question experimentally, using the spotless starling, *Sturnus unicolor*, in which exogenous testosterone in males increases territory size, polygyny (Veiga *et al.* 2001) and reproductive success (Veiga *et al.* 2002), although not EPFs (Cordero *et al.* 2003). We followed the same procedures as Veiga *et al.* (2001, 2002) and Cordero *et al.* (2003). We increased circulating testosterone in females by using implants with or without testosterone (T-females versus C-females). Then we used polymorphic microsatellites to test for rates of extra-pair paternity in their broods, assuming that EPFs contribute to variation in the breeding success of spotless starlings, as they do in many passerine species (Griffith *et al.* 2002). Testosterone implants in female spotless starlings are already known to delay the onset of laying and reduce clutch size, but they enhance their territorial behaviour and their survival, so T-females have similar lifetime reproductive success to C-females (Veiga & Polo, in press).

Although we do not know which sex solicits extra-pair copulations (EPCs) in the spotless starling, it is likely to be males, as they do in the closely related European starling, *Sturnus vulgaris* (Pinxten & Eens 1997). Thus if T-females look and behave more like males and are less attractive to them (Ketterson *et al.* 2005), potential extra-pair males should prefer C-females, which would therefore attain higher rates of EPFs than T-females. However, to our knowledge this is the first study on females in which EPOs have been analysed in relation to experimental testosterone manipulation. Because of the large effects of testosterone on female physiology, reproduction and behaviour (reviewed in Ketterson *et al.* 2005) we cannot discount the possibility that T-females encourage males to initiate EPCs. For example, song is stimulated by testosterone (e.g. Kern & King 1972; Arcese *et al.* 1988; Hausberger *et al.* 1995; Nespor *et al.* 1996), and T-females could use song as a display in the same way as males do to attract extra-pair mates (Catchpole & Slater 1995; Eens & Pinxten 1998). Furthermore, T-females could also become less selective about mates (McGlothlin *et al.* 2004), allowing more potential partners to engage in EPCs, thus increasing their chances of getting EPFs relative to C-females. Furthermore, we believe that variation in circulating testosterone in females could induce more complex changes than currently suspected. Thus, an analysis of rates of extra-pair paternity of C- and T-females would provide us with new information about female mating strategies, casting some light on the possible links between the evolution of male testosterone-dependent traits, levels of circulating testosterone in males and females, and patterns of mating from the female's perspective.

METHODS

Study Area and Species

The study was undertaken in a colony comprising a plot of 40 nestboxes of 1.3 ha at Villalba, near Madrid, Spain. The area is pastureland with scattered trees. A study of this colony has been underway since 1995 (Veiga *et al.* 2004).

The spotless starling is a facultatively polygynous passerine. Males are about 6% heavier than females. Egg laying extends from mid-April to mid-June and most females lay two clutches per breeding season (Mean = 4.9 eggs per clutch) in which hatching success varies between 65 and 71% of the eggs, and nestling mortality varies from 21 to 58% of the nestlings (Veiga 2002). Cordero *et al.* (2003) have documented EPO (16% of nestlings). Males and females defend nestboxes against competitors and intrasexual competition for nest sites is intense in both sexes.

Field Procedure

Paired females were captured in the nestboxes with guillotine spring traps before the onset of laying between late February and late March for the hormone experiment and blood sampling (50-100 µl). The capture of females did not affect either their mating status or their current nestbox occupation. The interval between the hormone treatment and the start of breeding for experimental females ranged from 2 to 88 days ($\overline{X} \pm SD = 44 \pm 21$ days). We ringed all adults with an aluminium ring and a combination of plastic colour rings to allow individual identification. Nestlings were ringed with aluminium rings when they were 5-6 days old and then bled. Blood was kept in the field in absolute ethanol and then stored at -40ºC until used to extract DNA. Nestlings were measured on the 16th day after hatching. The number of nestlings measured that day was considered to be the number of chicks fledged, as no ringed chick was found dead in the nestboxes after this day (Veiga *et al.* 2002).

We confirmed the social parental status of breeding females by capturing them when they fed nestlings or by identifying their colour rings. To assign social parental status of breeding males we observed the singing and territorial activity of colour-ringed individuals during prelaying and videorecorded them at each nest during incubation and nestling care (Moreno *et al.* 1999; Veiga *et al.* 2002). In 1996 and 1997 we validated these criteria by verifying that the presumed owners were the genetic fathers of at least some offspring in their nest by using DNA fingerprinting (Cordero *et al.* 2003).

The Hormone Experiment

During 1998, 1999 and 2000, we captured 58 females to be used as experimental females (T-females) or control females (C-females) depending on whether they received testosterone (T) or empty (C) implants. The T- and C-females were balanced according to age and experience. Of the females used in this study, 23 had previous breeding experience and 35 did not before manipulation. Of the former group, eight had a single year of breeding experience the year before manipulation (4 T- and 4 C-females), and 15 had 2 or more years of breeding experience before being implanted (7 T- and 8 C-females). Of the 35 females with no breeding experience, 19 were T-females and 16 were C-females. In total, we implanted 30 T- and 28 C-females. Females belonging to T or C groups did not differ statistically before manipulation in relation to any of the variables considered in this study, so they differed only in the level of testosterone.

The dose of testosterone we chose to administer was based on the finding that in some bird species, including the European starling (a sister species of the spotless starling), levels of circulating plasma testosterone are about four or five times lower in females than in males during the spring maximum (Silverin & Wingfield 1982; Balthazart 1983; Sandell, 2007). Thus, we used a fifth of the dose that we had used before to induce changes in territorial behaviour and parental investment in males of the same species with no adverse effect (Moreno *et al.*, 1999; Veiga *et al.* 2002). The T-females received a subcutaneous implant of a silastic tube (1.95 mm outer diameter, 1.47 mm inner diameter, 10 mm length; Dow Corning Iberica S.A., Barcelona, Spain) filled with testosterone propionate (Sigma Chemicals Co., St Louis, MI, U.S.A.), whereas C-females received an empty implant.

Implants were placed on the right shoulder, between the wings, while the birds were under anaesthesia (Forane, Abbot S.A., Madrid, Spain). The testosterone plasma concentration in similar, nearby experimental populations of the spotless starling treated with the same dose of testosterone was 0.36 ng/ml (range 0.04-1.08 ng/ml; *N*= 53) for C-females and 1.2 ± 0.55 ng/ml (range 0.13-2.79 ng/ml; *N*= 9; D. Gil & I. López, unpublished data) for T-females. The mean level recorded in T-females was similar to the maximum levels measured in non-manipulated females. These differences disappeared in the following year (D. Gil & I. López, unpublished data). Thus, the implants probably led to a relatively high level of testosterone in the circulating plasma of females.

After manipulation, 40 of 58 females bred either in the manipulation year or in the following years, the proportion being similar in T-females (19 of 30) and C-females (21 of 28; $X_1^2 = 0.92$, $P = 0.337$). Of the 40 breeding females, we used 38 for paternity analyses (18 T- and 20 C-females). Of these 38 females, 15 (4 T- and 11 C-females) bred only during the manipulation year and 23 (14 T- and 9 C-females) bred for 2 or more years. Females were implanted in 1998, 1999 and 2000 and we followed their reproduction until 2001. In the years after treatment we checked whether females maintained testosterone in their implants by their colour: full implants are white, losing this colour when empty.

Genetic Parentage Assignment

We carried out microsatellite genotyping on families whose social parentage was determined by continuous observation of individually ringed birds in the colony. We determined the true genetic relationships of 364 nestlings from 115 clutches of 38 breeding females and 41 putative social males. All genetic analyses of parental relationships were made blind with reference to treatment.

To extract and purify genomic DNA from the blood samples, we used NucleoSpin Tissue Kit (Macherey-Nagel, Duren, Germany). To determine parentage we used the following primers: Pca-7 from blue tit, *Cyanistes caeruleus* (Dawson *et* *al.* 2000), Ase-18 from Seychelles warbler, *Acrocephalus sechellensis* (Richardson *et al.* 2000), and SS2.32, SS1.6, SS1.106, SS.2.106 from superb starling, *Lamprotornis superbus* (Rubenstein 2005). These microsatellites were polymorphic for spotless starling and had a combined exclusion probability of 0.973 (García-Vigón *et al.* 2008).

We assessed genetic parentage by comparing nestling genotypes to those of the attending female and the territorial male at six loci. The alleles of within-pair offspring matched (i.e. they possessed compatible alleles with Mendelian inheritance) the social parents' alleles in all six loci or had one mismatched allele which we assumed originated through mutation in a given locus.

Young that mismatched the paternal allele at two or more loci were considered to be extra-pair offspring (EPO; e.g. Johnsen *et al.* 1998). Potential instances of pseudoparasitism (two or more mismatched loci in the social mother but all matching the social father) and intraspecific brood parasitism (both social parents mismatching in two or more loci) were also investigated

Data Analysis

To test for variation in rates of EPO between C- and T females we carried out an ANCOVA using the univariate option of general linear models of the SPSS statistical package, version 11.5 for Windows (SPSS Inc., Chicago, IL, U.S.A.). We did three different analyses: for the year of treatment, for all breeding seasons pooled and for the years after manipulation, excluding the year of treatment to detect possible long-term effects of testosterone on EPOs. The statistical unit for all ANCOVAS is the female, so we pooled all corresponding data per female and study period (year of manipulation, years after manipulation or total years) in each analysis in which the dependent variable was the percentage of EPO per female (number of EPO per female divided by the total number of nestlings analysed from that female per study period). This variable was arcsine square-root transformed (%EPO). To test for variation in %EPO in the year of manipulation, we included treatment (C or T) and year of manipulation (1998, 1999 or 2000) as fixed factors. To control for variables that were known to be or we suspected could be influential, we also included the following covariates: age of the female at the time of manipulation estimated as the number of years a female had bred in the colony before the manipulation year, date of laying of the first egg of the first clutch, total number of offspring produced that were bled and analysed, mating status of the female (i.e. whether she was mated with a monogamous or a polygynous male) and number of social mates obtained by the female / total reproductive attempts. We considered the latter to be a way of measuring a female's fidelity to her male during the period of study, because females may change mate in different breeding attempts and this could be associated with higher rates of EPO (Pinxten *et al.* 1993).

In the second analysis, we pooled all breeding attempts per female for all seasons including the year of manipulation. We followed the same protocol as before although we did not use the covariables laying date or female status. The covariable (nestlings) was measured as the sum of fledglings reared per female from the year of manipulation until the 2001 breeding season. The covariable (social mates) was measured as the total number of social mates obtained by females between the manipulation year and the 2001 breeding season divided by the total number of reproductive attempts from the year of manipulation until the 2001 breeding season.

Finally, we carried out a third analysis only for the years after manipulation to see whether there were any delayed effects of testosterone on the proportions of EPO. In this analysis, we also excluded the covariables laying date and female status. We did this analysis because some females did not breed in the year of manipulation, but did later. For this analysis we calculated average age, which represents the mean of all age values in the different years a female bred following treatment.

Initially each ANCOVA was constructed with all explanatory terms fitted, including interactions. Final models were selected following a backward procedure, by progressively eliminating nonsignificant variables. The significance of the remaining variables was tested again until no additional variable reached significance. All tests are two tailed and given at a significance level of 0.05.

Ethical Note

We bled nestlings at about 1 week of age, when they weighed 50 g or more, obtaining 50 µl of blood from the jugular vein. We noted no adverse effects, and their development was the same as in a control plot in which no blood was sampled. If the youngest chick was lighter than 50 g, we postponed sampling to a later visit. Adults sang and defended territories a few minutes after being bled (100 µl also from the jugular vein) and we never observed nest desertion or reduction in food provisioning or incubation bouts afterwards. Implant insertion caused no more apparent distress than blood sampling. Females were anaesthetized with Forane for less than 3 min and we found no immediate adverse effect after treatment or later on. There were no differences in the probability of breeding between C- and T-females in the experimental year ($X_1^2 = 0.02$, $P = 0.885$). The proportion of C- or T females breeding after treatment was similar in both groups (75% of C-females and 73.3% of T-females) suggesting that possible deleterious or adverse effects of testosterone were not affecting reproduction or survival negatively (see also Table 1 in Veiga *et al.* 2004 in which they include, among others, data of our studied females).

In addition, observational tracking of focal females during the days after manipulation showed that T-females behaved normally, although they were involved in aggressive encounters more frequently than C females. One year after implantation, females kept the implants while no trace of hormone (judged by implant colour) could be found. Spotless starlings are accustomed to human presence, as they normally breed in buildings, and the study colony has been monitored since 1995 with a high nestbox occupation and fairly good breeding success yearly. The manipulation of nests, ringing, bleeding and implant manipulation were undertaken under licence from the Spanish Ministry of the Environment (General Directorate for Nature

Conservation) and the Regional Government of Madrid.

RESULTS

For all years, 10.4% (38/364) of nestlings did not match their social father's genotype and 32.2% (37/115) of clutches contained at least one mismatched offspring. We found that 65% (13/20) of C-females and 27.8% (5/18) of T-females had at least one EPO in their nests ($X_1^2 = 5.26$, $P = 0.02$). In relation to nestlings, 15.3% (31/202) of C-females' chicks and 4.3% (7/162) of T-females' chicks were EPO ($X_1^2 = 11.69$, $P = 0.0006$). In relation to clutches: 48.2% (27/56) in C-females and 17% (10/58) in T-females had at least one EPO ($X_1^2 = 12.47$, $P < 0.001$). Finally, in the cases in which we could identify hatching order ($N = 43$), we found that there was not a biased distribution of extra-pair paternity among the youngest nestlings of the broods, which are likely to die (Yates corrected $X_1^2 = 0.05$, $P = 0.828$). Successful pseudoparasitism was rare (two instances, 0.55% of total nestlings; 0.58% of total clutches) and both occurred in a C-female's nest. We did not detect any case of intraspecific brood parasitism.

Testosterone and EPO in the Manipulation Year

After controlling for other influential variables, we found that treatment affected %EPO, with T-females showing lower rates of EPO than C-females ($F_{1,24} = 8.937$, $P = 0.006$; $Fig.1$). This difference was not constant across the 3 years of manipulation (interaction treatment*manipulation year: $F_{4,24} = 4.251$, $P = 0.011$). Other significant variables entering the model and associated with %EPO were female age, with older females showing lower rates of EPO than younger females ($F_{1,24} = 4.393$, $P = 0.047$) and nestlings, which was positively associated with %EPO ($F_{1,24} = 9.961$, $P = 0.004$). The other variables (laying date, year, social mates, female status) and their interactions were nonsignificant (all $P > 0.05$) so they were excluded from the final model ($F_{7,24} = 4.80$, $P = 0.002$)

Long-Term Effects of Testosterone on EPOs

The difference in %EPO between T- and C-females was highest when we pooled all breeding seasons per female ($F_{1,31} = 9.394$, $P = 0.004$, *Fig.*1) suggesting that effects of testosterone may persist beyond the manipulation year.

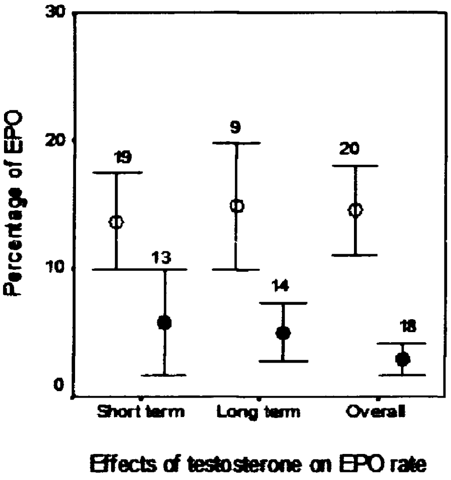

Figure 1. Percentage of extra-pair offspring (EPO) for control (•) and testosterone-treated (o) female spotless starlings in relation to the time elapsed since testosterone manipulation. The effect of testosterone manipulation on EPO rate is shown for the manipulation year (short term), in the years after manipulation (long term) and in all years and breeding attempts by females, including females not breeding in the year of manipulation but doing so later (overall). Means are shown ± 1SE. The number of sampled females is given above the points.

Female age in the manipulation year was associated with %EPO, with younger females having higher rates of EPO than older ones ($F_{1,31} = 9.671$, $P = 0.004$; *Fig.*3). No other variable (nestlings, social mates, year), or their interactions, was significant (all $P > 0.05$; final model: $F_{1,31} = 6.47$, $P < 0.001$).

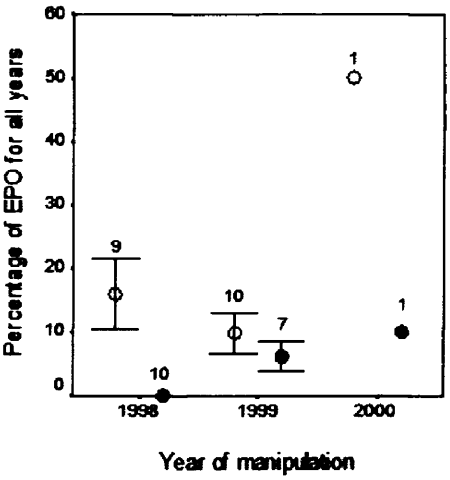

Figure 2. Percentage of extra-pair offspring (EPO) for control (o) and testosterone-treated (•) female spotless starlings for all years and breeding attempts indicating the year in which females were manipulated. Means are shown ± 1SE. The number of sampled females is given above the points.

Female age in the manipulation year was associated with %EPO, with younger females having higher rates of EPO than older ones ($F_{1,31} = 9.671$, $P = 0.004$; *Fig.*3). No other variable

(nestlings, social mates, year), or their interactions, was significant (all $P > 0.05$; final model: $F_{1,31} = 6.47$, $P < 0.001$).

To see the importance of delayed effects of treatment on %EPO, we carried out a new analysis excluding data from the year of manipulation. We found that treatment was still significant ($F_{1,20} = 4.520$, $P = 0.046$; *Fig*.1) indicating a long-term effect of testosterone on reducing %EPO beyond the year of treatment even though implants were already empty. In addition, average age was again negatively associated with %EPO ($F_{1,20} = 6.819$, $P = 0.017$).

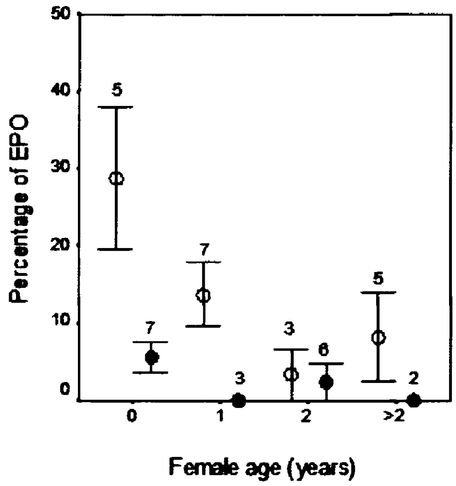

Figure 3. Percentage of extra-pair offspring (EPO) for control (○) and testosterone-treated (●) female spotless starlings in relation to female age in the manipulation year and considering all breeding attempts of females. Means are shown ± 1SE. The number of sampled females is given above the points.

DISCUSSION

Female spotless starlings treated with testosterone significantly reduced the percentage of EPO in relation to controls. As far as we know, this is the first study relating an experimental increase of circulating levels of testosterone and proportions of EPO in a female bird. Although our manipulating protocol was uniform in the 3 years of the experiment, we obtained a significant interaction between treatment and manipulation year. This interaction reflected different results between years with 1998 showing a clearer pattern than 1999 that is difficult to explain. We repeated the manipulation experiment in different years and with different females to enlarge the sample size, so relatively small sample sizes per season and across years could cause spurious interactions with year that may cloud the main picture. However, we were interested in the general effects of female treatment on rates of EPO and we believe that the overall result is valid in spite of this interaction. Furthermore, the difference between C- and T-females in relation to EPO became more consistent when we considered all breeding attempts per female for the whole period of study. Our results showed that the effect of treatment on rates of EPO persisted in the following breeding seasons (1-3 years after manipulation) when the experimental implants were empty, reinforcing our main finding of the relation between treatment and rates of EPO. Long-term effects of exogenous testosterone have also been found in other studies (Collis & Borgia 1992; Schwabl 1993; Ros *et al.* 1997) including male (Veiga *et al.* 2001) and female (Veiga *et al.* 2004) spotless starlings. These effects may be related to irreversible physiological changes in females after treatment (Staub & De Beer 1997) or to changes in the social status of the treated females (Veiga *et al.* 2004). One possible explanation for differences in rates of EPO between T- and C-females could be related to differential rates of mortality between intra- and extra-pair offspring according to their hatching order. However, we can discard this possibility because instances of EPO were not particularly biased to the youngest nestling, which are thought to have a higher probability of dying from starvation.

In contrast to studies on males, our results do not support the hypothesis that testosterone might enhance mating and courtship behaviour in females (Lank *et al.* 1999; McGlothlin *et al.* 2004), at least in relation to extra-pair matings. The T-females were less successful at extra-pair mating than the C-females, perhaps because exogenous testosterone masculinized both morphological and behavioural traits of experimental females making them more like males (see instances in Ketterson *et al.* 2005). This would have made them less attractive to potential extra-pair mates, assuming that males are the sex promoting EPCs in the spotless starling as they are in the closely related European starling (Pinxten & Eens 1997) and in other avian species (e.g. Magrath & Elgar 1997).

Spotless starling females could have fewer EPO simply because they invest more time in aggressive encounters maintaining or gaining a new territory (see Sandell 2007 for European starling) although other phenotypic or behavioural changes may also be decisive as mentioned above. Alternatively, T-females, because of their more aggressive behaviour, may not allow extra-pair mating, a hypothesis requiring further study. The fact that extra-pair males are more likely to have EPO with younger than with older females is interesting as most studies on promiscuous mating have rarely examined the female's perspective (see, however, Dickinson 2001). Although the relation between female age and percentage of EPO was independent of treatment (interaction treatment * female age was not significant, $Ps > 0.3$), this result suggests that testosterone could change the behaviour of young females so that they appear older. Irrespective of direct or indirect testosterone-related effects on female birds, we still have many unsolved theoretical and empirical questions on the mechanisms, causes and consequences of extra-pair paternity, a phenomenon that is very variable between bird species (Petrie & Kempenaers 1998; Griffith *et al.* 2002; Westneat & Stewart 2003). As female spotless starlings manipulated with exogenous testosterone had fewer EPOs than controls, it would be interesting to investigate whether different levels of circulating testosterone in females of natural populations across different taxa follow the same pattern and are associated with different rates of EPO. Levels of testosterone in females of different bird species could be a correlative by product of selection of a testosterone-dependent trait in males (McGlothlin *et al.* 2004; Ketterson *et al.* 2005; Møller *et al.* 2005). It could also be the product of direct selection on females, and in this case testosterone should covary with female ecology between species (Ketterson *et al.* 2005). To see whether our findings on spotless starlings could be extended to other species, we could look for studies where circulating levels of testosterone and rates of EPO have been analysed separately. For example, relatively high levels of female testosterone may be found in (1) species in which both males and females have high levels such as colonial bird species (Møller *et al.* 2005) and (2) species in which only females have high levels such as sexually monomorphic species and species with socially monogamous mating systems (Ketterson *et al.* 2005). However, data on EPO are diverse and contradictory in this respect as EPO are expected to be higher among dimorphic than monomorphic species (Møller & Birkhead 1994) and among those attaining higher densities than solitary breeders (Westneat & Sherman 1997; Møller & Ninni 1998).

In conclusion, we found for the first time that an exogenous administration of testosterone in female birds (spotless starling) reduces proportions of EPO. Furthermore, but not less importantly, we have shown that this difference persists in individuals for several years after manipulation. It would be of interest to carry out more contrasting experiments in this respect and in different species and with reproductive strategies other than facultative polygyny to see the possible repercussions and generalization of the effects of testosterone on alternative mating tactics of females in other vertebrate species.

ACKNOWLEDGMENTS

We thank Marta Arenas, Sara Sánchez and Javier Viñuela for their help during the field work. We also acknowledge Joaquín Ortego and José Miguel Aparicio for their inestimable help with Excel macros, particularly the program Cernicalín (Aparicio *et al.* 2006) for genetic analyses. We are also grateful to Vicente Polo for his comments and discussions on the manuscript, and finally, many thanks to Ben Hatchwell and three anonymous referees for their constructive comments and suggestions. During this work E.G-V. was supported by a predoctoral fellowship from the Ministerio de Educación y Ciencia. This research was funded by project CGL-2005-05611-C02-01 and 02/ BOS of the Ministerio de Educación y Ciencia.

REFERENCES

Adkins-Regan, E. 1999. Testosterone increases singing and aggression but not male-typical sexual partner preference in early estrogen treated female zebra finches. *Hormones and Behavior*, **35**: 63-70.

Aparicio, J. M., Ortego, J. & Cordero, P. J. 2006. What should we weigh to estimate heterozygosity, alleles or loci?. *Molecular Ecology*, **15**: 4659-4665.

Arcese, P., Stoddard, P. & Hierbert, S. 1988. The form and function of song in female song sparrows. *Condor*, **90**: 44-50.

Arnold, A. P. 2004. Sex-chromosomes and brain gender. *Nature Reviews Neuroscience*, **5**: 701-708.

Balthazart, J. 1983. Hormonal correlates of behaviour. In: *Avian Biology*. Vol. 7 (Ed. by D. S. Farner, J. R. King & K. C. Parkes), pp. 221-366. New York: Academic Press.

Beletsky, L. D., Gori, D. F., Freeman, S. & Wingfield, J. C. 1995. Testosterone and polygyny in birds. *Current Ornithology*, **12**: 1-41.

Buchanan, K. L., Evans, M. R., Goldsmith, A. R., Bryant, D. M. & Rowe, L. V. 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling?. *Proceedings of the Royal Society of London, Series B*, **268**: 1337-1344.

Casto, J. M., Nolan, V., Jr. & Ketterson, E. D. 2001. Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *American Naturalist*, **157**: 408-420.

Catchpole, C. K. & Slater, P. J. B. 1995. *Bird Song: Biological Themes and Variations*. New York: Cambridge University Press.

Clotfelter, E.D., O'Neal, D.M., Gaudioso, J.M., Casto, J.M., Parker- Renga, I. A., Snajdr, E. A., Duffy, D. L., Nolan, V., Jr. & Ketterson, E. D. 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution?. *Hormones and Behavior*, **46**: 171-179.

Collis, K. & Borgia, G. 1992. Age-related effects of testosterone, plumage, and experience on aggression and social dominance in juvenile male satin bowerbirds (*Ptilonorhynchus violaceus*). *Auk*, **109**: 422-434.

Cordero, P. J., Veiga, J. P., Moreno, J. & Parkin, D. T. 2003. Extrapair paternity in the nest of the facultatively polygynous spotless starling (*Sturnus unicolor*). *Behavioral*

Ecology and Sociobiology, **54**: 1-6.

Dawson, D. A., Hanotte, O., Greig, C., Stewart, I. R. K. & Burke, T. 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. *Molecular Ecology*, **9**: 1941-1943.

De Ridder, E., Pintxen, R., Mees, V. & Eens, M. 2002. Short and long term effects of male like concentrations of testosterone on European starlings (*Sturnus vulgaris*). *Auk*, **119**: 487-497.

Desjardins, C. & Turek, F. W. 1977. Effects of testosterone on spermatogenesis and luteinizing hormone release in Japanese quail. *General and Comparative Endocrinology*, **33**: 293-303.

Dickinson, J. L. 2001. Extrapair copulations in western bluebirds (*Sialia mexicana*): female receptivity favors older males. *Behavioral Ecology and Sociobiology*, **50**: 423-429.

Duffy, D. L., Bentley, G. E., Drazen, D. L. & Ball, G. F. 2000. Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. *Behavioral Ecology*, **11**: 654-662.

Dufty, A. M., Jr. 1989. Testosterone and survival: a cost of aggressiveness? *Hormones and Behavior*, **23**: 185-193.

Eens, M. & Pinxten, R. 1998. Female song for male attraction: an overlooked phenomenon?. *Trends in Ecology & Evolution*, **13**: 322-323.

Eens, M., Van Duyse, E., Bergman, L. & Pinxten, R. 2000. Shield characteristics are testosterone-dependent in both male and female moorhens. *Hormones and Behavior*, **37**: 126-134.

Enstrom, D. A., Ketterson, E. D. & Nolan, V., Jr. 1997. Testosterone and mate choice in the dark-eyed junco. *Animal Behaviour*, **54**: 1135-1146.

Feuerbacher, I. & Prinzinger, R. 1981. The effects of the male sex-hormone testosterone on body temperature and energy metabolism in male Japanese quail (*Coturnix japonica*). *Comparative Biochemistry and Physiology*, **70**A: 247-250.

Foerster, K. & Kempenaers, B. 2004. Experimentally elevated plasma levels of testosterone do not increase male reproductive success in blue tits. *Behavioral Ecology and Sociobiology*, **56**: 482-490.

Folstad, I. & Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. *American Naturalist*, **139**: 603-622.

Garamszegi, L. Z., Eens, M., Hurtrez-Bousse` s, S. & Møller, A. P. 2005. Testosterone, testes size, and mating success in birds: a comparative study. *Hormones and Behavior*, **47**: 389-409.

García-Vigón, E., Cordero, P. J. & Veiga J. P. 2008. Cross-amplified polymorphic microsatellites for the spotless starling *Sturnus unicolor*. *Ardeola*, **55**: 3-11.

Griffith, S. C., Owens, I. P. F. & Thuman, K. A. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**: 2195-2212.

Hannsler, I. & Prinzinger, R. 1979. The influence of the sex hormone testosterone on body temperature and metabolism of Japanese quail. *Experientia*, **35**: 509-510.

Hausberger, M., Henry, L. & Richard, M. 1995. Testosterone induced singing in female starlings (*Sturnus vulgaris*). *Ethology*, **99**: 193-208.

Hillgarth, N. & Wingfield, J. C. 1997. Parasite-mediated sexual selection: endocrine aspects. In: *Hoste Parasite Evolution: General Principles and Avian Models* (Ed. by D. H. Clayton & J. Moore), pp. 78-104. New York: Oxford University Press.

Hirschenhauser, K. & Oliveira, R. F. 2006. Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. *Animal Behaviour*, **71**: 265-277.

Hunt, K. E., Hahn, T. P. & Wingfield, J. C. 1997. Testosterone implants increase song but not aggression in male Lapland longspurs. *Animal Behaviour*, **54**: 1177-1192.

Johnsen, A., Andersson, S., Ornborg, J. & Lifjeld, J. T. 1998. Ultraviolet plumage ornamentation affects social mate choice and sperm competition in bluethroats. *Proceedings of the*

Royal Society of London, Series B, **265**: 1313-1318.

Kast, T. L., Ketterson, E. D. & Nolan, V., Jr. 1998. Variation in ejaculate quality in dark-eyed juncos according to season, stage of reproduction, and testosterone treatment. *Auk*, **115**: 684-693.

Kern, M. & King, J. 1972. Testosterone-induced singing in female white-crowned sparrows. *Condor*, **74**: 204-209.

Ketterson, E. D. & Nolan, V., Jr. 1994. Male parental behaviour in birds. *Annual Review of Ecology and Systematics*, **25**: 601-628.

Ketterson, E. D. & Nolan, V., Jr. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *American Naturalist*, **154**: S4-S25.

Ketterson, E. D., Nolan, V., Jr., Wolf, L., Ziegenfus, C., Duffy, D. L., Ball, G. F. & Johnsen, T. S. 1991. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos (*Junco hyemalis*). *Hormones and Behavior*, **25**: 489-503.

Ketterson, E. D., Nolan, V., Jr., Casto, J. M., Buerkle, C. A., Clotfelter, E., Grindstaff, J. L., Jones, K. J., Lipar, J. L., McNabb, F. M. A., Neudorf, D. L., Parker-Renga, I., Schoech, S. J. & Snajdr, E. 2001. Testosterone, phenotype and fitness: a research program in evolutionary behavioural endocrinology. In: *Avian Endocrinology* (Ed. by A. Dawson & C. M. Chaturvedi), pp. 19-40. New Delhi: Narosa Publishing House.

Ketterson, E. D., Nolan, V., Jr. & Sandell, M. 2005. Testosterone in females: mediator of adaptive traits, constraint on sexual dimorphism, or both?. *American Naturalist*, **166**: 85-98.

Lank, D., Coupe, M. & Wynne-Edwards, K. E. 1999. Testosterone induced male traits in female ruffs (*Phylomachus pugnax*): autosomal inheritance and gender differentiation. *Proceedings of the Royal Society of London, Series B*, **266**: 2323-2330.

McGlothlin, J. W., Neudorf, D. L. H., Casto, J. M., Nolan, V., Jr. & Ketterson, E. D. 2004. Elevated testosterone reduces choosiness in female dark eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? *Proceedings of the Royal Society of London, Series B*, **271**: 1377-1384.

Magrath, M. J. L. & Elgar, M. A. 1997. Paternal care declines with increased opportunity for extra-pair matings in fairy martins. *Proceedings of the Royal Society of London, Series B*, **264**: 1731-1736.

Møller, A. P. & Birkhead, T. R. 1994. The evolution of plumage brightness in birds is related to extra-pair paternity. *Evolution*, **48**: 1089-1100.

Møller, A. P. & Ninni, P. 1998. Sperm competition and sexual selection: a meta-analysis of paternity studies of birds. *Behavioral Ecology and Sociobiology*, **43**: 345-358.

Møller, A. P., Garamszegi, L. Z., Gil, D., Hurtrez-Bousse` s, S. & Eens, M. 2005. Correlated evolution of male and female testosterone profiles in birds and its consequences. *Behavioral Ecology and Sociobiology*, **58**: 534-544.

Moore, M. C. 1984. Changes in territorial defence produced by changes in circulating levels of testosterone: a possible hormonal basis for mate guarding behaviour in white-crowned sparrows. *Behaviour*, **88**: 215-226.

Moreno, J., Veiga, J. P., Cordero, P. J. & Mínguez, E. 1999. Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **47**: 47-53.

Mougeot, F., Irvine, J. R., Seivwrigth, L., Redpath, S. M. & Piertney, S. 2004. Testosterone, immunocompetence, and honest sexual signalling in male red grouse. *Behavioral Ecology*, **15**: 215-226.

Nelson, R. J. 2000. *An Introduction to Behavioral Endocrinology*. 2nd edn. Sunderland, Massachusetts: Sinauer.

Nespor, A., Lukazewicz, M., Dooling, R. & Ball, G. 1996. Testosterone induction of male-like vocalizations in female budgerigars (*Melopsittacus undulatus*). *Hormones and Behavior*, **30**: 162-169.

Nolan, V., Jr., Ketterson, E. D., Ziegenfus, D. P., Cullen, D. P. & Chandler, C. R. 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on prebasic molt and survival in male dark-eyed juncos. *Condor*, **94**: 364-370.

Owen-Ashley, N. T., Hasselquist, D. & Wingfield, J. C. 2004. Androgens and the immunocompetence handicap hypothesis: unravelling direct and indirect pathways of immunosuppression in song sparrows. *American Naturalist*, **164**: 490-505.

Petrie, M. & Kempenaers, B. 1998. Extra-pair paternity in birds: explaining variation between species and populations. *Trends in Ecology & Evolution*, **13**: 52-57.

Pinxten, R. & Eens, M. 1997. Copulation and mate-guarding patterns in polygynous European starlings. *Animal Behaviour*, **54**: 45-58.

Pinxten, R., Hanotte, O., Eens, M., Verheyen, R. F., Dhondt, A. A. & Burke, T. 1993. Extra-pair paternity and intraspecific brood parasitism in the European starling, *Sturnus vulgaris*: evidence from DNA fingerprinting. *Animal Behaviour*, **45**: 795-809.

Raouf, S. A., Parker, P. G., Ketterson, E. D., Nolan, V. & Ziegenfus, C. 1997. Testosterone affects reproductive success by influencing extrapair fertilizations in male dark-eyed juncos. *Proceedings of the Royal Society of London, Series B*, **264**: 1599-1603.

Reed, W. L., Clarck, M. E., Parker, P. G., Raouf, S. A., Arguedas, N., Monk, D. S., Snajdr, E., Nolan, V., Jr. & Ketterson, E. D. 2006. Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. *American Naturalist*, **167**: 667-683.

Richardson, D. S., Jury, F. L., Dawson, D. A., Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in *Sylviidae* species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**: 2226-2231.

Roberts, M. L., Buchanan, K. L. & Evans, M. R. 2004. Testing the immunocompetence handicap hypothesis. *Animal Behaviour*, **68**: 227-239.

Ros, A. F. H., Groothuis, T. G. G. & Apanius, V. 1997. The relation among gonadal steroids, immunocompetence, body mass, and behaviour in young black-headed gulls (*Larus ridibundus*). *American Naturalist*, **150**: 201-219.

Rubenstein, D. R. 2005. Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*. *Molecular Ecology Notes*, **5**: 739-744.

Rutkowska, J., Cichon, M., Puerta, M. & Gil, D. 2005. Negative effects of elevated testosterone on female fecundity in zebra finches. *Hormones and Behavior*, **47**: 585-591.

Sandell, M. I. 2007. Exogenous testosterone increases female aggression in the European starling. *Behavioral Ecology and Sociobiology*, **62**: 255-262.

Schlinger, B. A., Soma, K. K. & Saldanha, C. 2001. Perspectives in ornithology: advances in avian behavioral endocrinology. *Auk*, **118**: 283-289.

Schoech, S. J., Ketterson, E. D., Nolan, V., Jr., Sharp, P. J. & Buntin, J. D. 1998. The effect of exogenous testosterone on parental behavior, plasma prolactin, and prolactin binding sites in dark-eyed juncos. *Hormones and Behavior*, **34**: 1-10.

Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences of U.S.A.*, **90**: 11446-11450.

Searcy, W. A. 1988. Do female red-winged blackbirds limit their own breeding densities?. *Ecology*, **69**: 85-95.

Silverin, B. & Wingfield, J. C. 1982. Patterns of breeding behaviour and plasma-levels of hormones in a free-living population of pied flycatchers, *Ficedula hypoleuca*. *Journal of Zoology*, **198**: 117-129.

Staub, N. L. & De Beer, M. 1997. The role of androgens in female vertebrates. *General and*

Comparative Endocrinology, **108**: 1-24

Veiga, J. P. 2002. Estornino negro: *Sturnus unicolor*. In: *Enciclopedia Virtual de los Vertebrados Españoles* (Ed. by L. M. Carrascal & A. Salvador), pp. 1-22. Madrid: Museo Nacional de Ciencias Naturales.

Veiga, J. P. & Polo, V. In press. Fitness consequences of increased testosterone levels in females spotless starling. *American Naturalist.*

Veiga, J. P., Moreno, J., Cordero, P. J. & Mínguez, E. 2001. Territory size and polygyny in the spotless starling: resource-holding potential or social inertia. *Canadian Journal of Zoology*, **79**: 1951-1956.

Veiga, J. P., Moreno, J., Arenas, M. & Sánchez, S. 2002. Reproductive consequences for males of paternal vs territorial strategies in the polygynous spotless starling under variable ecological. *Behaviour*, **139**: 677-693.

Veiga, J. P., Viñuela, J., Cordero, P. J., Aparicio, J. M. & Polo, V. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. *Hormones and Behavior*, **46**: 47-53.

Watson, A. & Parr, R. 1981. Hormone implants affecting territory size and aggressive and sexual behaviour in red grouse. *Ornis Scandinavica*, **12**: 55-61.

Westneat, D. F. & Sherman, P. W. 1997. Density and extra-pair fertilizations in birds: a comparative analysis. *Behavioral Ecology and Sociobiology*, **41**: 205-215.

Westneat, D. F. & Stewart, I. R. K. 2003. Extra-paternity in birds: causes, correlates, and conflicts. *Annual Review of Ecology, Evolution and Systematics*, **34**: 365-396.

Wingfield, J. C. 1984. Androgens and mating systems: testosterone induced polygyny in normally monogamous birds. *Auk*, **101**: 665-671.

Wingfield, J. C. 1994. Hormone-behaviour interactions and mating systems in male and female birds. In: *The Differences Between the Sexes* (Ed. by R. V. Short & E. Balaban), pp. 303-330. Cambridge: Cambridge University Press.

Wingfield, J. C., Hegner, R. E., Dufty, A. M., Jr. & Ball, G. F. 1990. The 'challenge hypothesis': theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136**: 829-846.

Wingfield, J. C., Lynn, S. E. & Soma, K. K. 2001. Avoiding the 'cost' of testosterone: ecological bases of hormone-behaviour interactions. *Brain, Behaviour and Evolution*, **57**: 239-251.

Zysling, D. A., Greives, T. J., Breuner, C. W., Casto, J. M., Demas, G. E. & Ketterson, E. D. 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis carolinensis*). *Hormones and Behavior*, **50**: 200-207.

CAPÍTULO 3

La tasa de ceba del macho y la paternidad extra-pareja en el estornino negro con poliginia facultativa

Elena García-Vigón, José Pablo Veiga & Pedro J. Cordero. 2009.
Male feeding rate and extra-pair paternity in the facultatively polygynous spotless starling. *Animal Behaviour*, **78**: 1335-1341.

RESUMEN

En aves, los machos se enfrentan a menudo a una pérdida de paternidad en sus nidos como consecuencia del comportamiento promiscuo de sus parejas. Según la teoría de inversión parental el cuidado de pollos de machos rivales tiene costes, de manera que la selección actúa en contra de los machos que cuidan de aquellos pollos con los que no están relacionados genéticamente. En este estudio examinamos la relación entre el cuidado parental y la paternidad en el estornino negro, *Sturnus unicolor*, durante tres estaciones de cría consecutivas. Nosotros no encontramos evidencias de que los machos de estornino negro redujesen la tasa de ceba a los pollos en función de su pérdida de paternidad en los nidos. Sorprendentemente, los machos que perdían paternidad en sus nidos tuvieron tasas de ceba más altas en ellos que los machos que no perdieron paternidad. Aunque este resultado merece mas investigación, nosotros argumentamos que los machos de estornino negro no reducen su tasa de ceba cuando pierden paternidad principalmente porque el hecho de variar la tasa de ceba no es muy costoso en una especie en la que la poligamia esta relacionada con la eficacia biológica del macho, la ocurrencia de paternidad extra-pareja es impredecible y las claves fiables para evaluar la paternidad son poco probables. Además presentamos varias hipótesis para interpretar un incremento en la tasa de ceba de los machos que pierden paternidad respecto a los machos que no pierden paternidad en términos de calidad parental, concibiendo las cebas como una forma de esfuerzo de apareamiento o relacionadas con la selección de parientes y dependientes del nivel de petición de los pollos ("begging"), la competicición entre hermanos y el parentesco genético dentro de las polladas.

Palabras clave: paternidad extra-pareja, tasa de ceba, microsatélites, cuidado parental, *Sturnus unicolor*.

Male feeding rate and extra-pair paternity in the facultatively polygynous spotless starling

ABSTRACT

Male birds often face a loss of paternity in the broods they attend because of promiscuous behaviour by their mates. Parental investment theory predicts that caring for the offspring of rival males incurs costs, selecting against males that care for unrelated offspring. We examined the relationship between male parental care and paternity in the facultatively polygynous spotless starling, *Sturnus unicolor*, during three consecutive breeding seasons. We found no evidence that male spotless starlings reduce their feeding rates to their nestlings in relation to their loss of paternity. Unexpectedly, cuckolded males provisioned nestlings more than noncuckolded males in relation to the presence of extra-pair offspring. Although this result deserves further investigation, we argue that spotless starling males do not reduce their feeding rate when they lose paternity mostly because variance in male feeding rate is not very costly in a species in which polygamy is related to male fitness, the occurrence of extra-pair offspring is unpredictable and reliable cues to assess paternity are unlikely. Several hypotheses are presented to interpret an increase in feeding rates of cuckolded males over non-cuckolded males in terms of parental quality with feeding rates either envisaged as a form of mating effort or related to kin selection and depending on begging level, sibling competition and genetic parentage within broods.

Keywords: extra-pair paternity, feeding rate, microsatellite, paternal care, *Sturnus unicolor*.

INTRODUCTION

Molecular paternity analyses have revealed that extra-pair paternity is a widespread phenomenon among birds (Westneat *et al.* 1990; Birkhead & Møller 1992; Griffith *et al.* 2002). So broods with mixed paternity in which the attending male sires only some, if any, of the young may be the norm in a high proportion of species (reviewed in Westneat & Webster 1994). How cuckolded males respond in terms of parental care when facing a loss of paternity in their nests has been a question of substantial interest (MacDougall- Shackleton & Robertson 1998). Traditional parental investment theory predicts that animals that provide parental care should be selected to avoid provisioning unrelated offspring (Trivers 1972). This topic has been explored in several species (reviewed in Whittingham & Dunn 2001) and many studies have found an adjustment of paternal care in response to perceived paternity (e.g. Dixon *et al.*1994; Weatherhead *et al.* 1994; Sheldon & Ellegren 1998; Lifjeld *et al.* 1998; Chuang-Dobbs *et al.* 2001; Neff 2003). However, many other studies relating paternity (or certainty of paternity) to parental care have failed to detect such an adjustment (e.g. Davies 1992; Wagner 1992; Whittingham *et al.* 1993, 2003; Westneat 1995; Whittingham & Lifjeld 1995; Yezerinac et al.1996; Wagner *et al.* 1996; Kempenaers *et al.*1998; MacDougall-Shackleton & Robertson 1998; Peterson *et al.* 2001; Dickinson 2003; Bouwman *et al.* 2005; see also Kempenaers & Sheldon 1997 & references therein). An explanation for these conflicting results may be found in species-specific differences in promiscuous and parental behaviours and their trade-offs. Theoretical models describe the specific conditions under which males should adjust their parental effort in response to level of paternity (Werren *et al.* 1980; Whittingham *et al.* 1992; Xia 1992; Westneat & Sherman 1993; Houston 1995; Kokko 1999). These models predict that the facultative adjustment of paternal effort in relation to male share of paternity may occur when (1) levels of extra-pair offspring (EPO) vary between breeding attempts of the same male in a predictable manner, allowing males with low levels of paternity to achieve higher reproductive success in other broods, (2) males have some cues that allow them to recognize the presence of EPO in their nest and (3) the benefits of reducing paternal care offset the costs (Westneat & Sherman 1993). To understand the relationship between male parental effort and paternity, one must consider all of these key factors because the absence of any is likely to prevent parental adjustment in response to reduced paternity (Whittingham *et al.* 1992; Westneat & Sherman 1993). We examined the relationship between male parental care and paternity in the facultatively polygynous spotless starling, *Sturnus unicolor*. The spotless starling is a good subject to explore the relationship between male provisioning and paternity because its characteristics allow us to test the different assumptions of theoretical models for the adjustment of paternal care in relation to the level of paternity. First, the spotless starling is a double-brooded species with a relatively long life. This allows us to study the pattern of parentage in successive breeding attempts of the same pair between years and within breeding seasons, which is important in determining whether males will benefit or not from providing paternity-dependent care (Yezerinac *et al.* 1996). Second, certain levels of extra-pair paternity have been reported for this species (16–20% of the nestlings were EPO; Cordero *et al.* 2003; García-Vigón *et al.* 2008a, b). Therefore, it is possible to test for the existence of cues allowing male spotless starlings to recognize the presence of EPOs, by comparing male feeding rates in broods of cuckolded and non cuckolded males. Finally, facultative polygyny, philopatry and biparental care all occur in the spotless starling and may affect the trade-offs of male parental care adjustment according to levels of paternity. For example, the degree of polygyny may affect the extent of paternal care (Lack 1968) because male reproductive effort has to be allocated to parental effort or mating effort (Maynard Smith 1977). In the spotless starling, the varying selective forces acting on breeding males seem to operate more frequently against the development of parental care, as the polygynous strategy is favoured under a wide array of ecological conditions (Veiga *et al.* 2002). Westneat & Sherman (1993) have suggested that in polygynous species, the relationship between the parentage of offspring in the current breeding attempt and the parentage of offspring from additional matings is important for the adjustment of parental care in relation to the male's level of paternity (Westneat & Sherman 1993). In spotless starlings, the parentage of offspring in the current breeding attempt and the parentage of offspring from additional matings are similar (E. García-Vigón, P.J. Cordero & J.P. Veiga, unpublished data). Therefore, and taking into account the assumptions of theoretical models and the behavioural breeding tactics of the spotless starling, we started from the contention that male spotless starlings should not adjust their feeding rates in relation to the level of EPO in their broods. To test this hypothesis, we analysed (1) food provisioning rates to nestlings by males and females and (2) levels of paternity by polymorphic microsatellite genotyping in colony of spotless starlings in which birds had been individually identified and monitored since 1995.

METHODS

Species and Study Area

Male and female spotless starlings defend nestboxes against competitors and intrasexual competition for nest sites is heavy in both sexes. Egg laying extends from mid-April to mid-June and most females lay two clutches per breeding season. Most of the incubation is done by the female (80–100% of the time). Nestlings are fed by both sexes, although secondary females paired with polygynous males have to do nearly all the work (Veiga *et al.* 2002). Male contribution to provisioning is important in the early breeding period (Moreno *et al.* 1999) although female condition may be crucial for the survival of the offspring (Moreno *et al.* 2002). We descrite in the Data analysis section how we analysed feeding rate data. We studied spotless starlings in 1998, 1999 and 2000 in a colony consisting of 40 nestboxes in a plot of 1.3 ha at Villalba, central Spain (40_300N, 4_W). The area is pastureland with scattered trees. A study of this colony has been underway since 1995 (Veiga *et al.* 2004). Nestboxes were checked to determine the beginning of laying, clutch size and hatching date. On day 16 after hatching, nestlings were ringed with aluminium rings and tarsus length, wing length and body mass were measured. Adults were caught in the nestboxes with spring traps and they were ringed with an aluminium ring and a combination of plastic colour rings to allow individual identification. The capture of males and females did not affect either their mating status or their current nestbox occupation. Blood samples were taken from the jugular vein of all adults and 4–6 day-old nestlings, kept in absolute ethanol and stored at -40 °C until they were used for DNA analysis.

Parentage Assignment

The identity of social females was determined by capturing them when they fed nestlings or from their colour rings. The identity of social males was determined by conducting observations of song and territorial activity of colour-ringed individuals during prelaying and by videotape recordings at each nest during incubation and nestling care (Moreno *et al.* 1999; Veiga *et al.* 2002). In 1996 and 1997, we validated this criterion by verifying that the presumed owners were the genetic fathers of at least some offspring in their nest using DNA fingerprinting (Cordero *et al.* 2003). To establish the true genetic relationships in the years included in this study, we carried out microsatellite genotyping on families whose social parentage had been determined. To extract and purify genomic DNA from the blood samples we used NucleoSpin Tissue Kit (Macherey-Nagel, Duren, Germany). We determined the genetic parentage for 34 social males, 41 breeding females and 327 nestlings from 92 broods using the following primers: Pca-7 from blue tit, *Cyanistes caeruleus* (Dawson *et al.* 2000), Ase-18 from Seychelles warbler, *Acrocephalus sechellensis* (Richardson *et al.* 2000) and SS2.32, SS1.6, SS1.106, SS2.106 from superb starling, *Lamprotornis superbus* (Rubenstein 2005). These microsatellites were polymorphic for spotless starling with an average of eight alleles per locus (range 4–12) and had a combined exclusion probability of 0.973. Their suitability for paternity analyses has been validated in two previous studies (García-Vigón *et al.* 2008a, b). We assessed genetic parentage by comparing nestling genotypes first to those of the attending female and then to those of the territorial male at six loci. We considered that nestlings were within-pair offspring (WPO) if their alleles matched (i.e. if they possessed alleles of the size of each parent and segregation was compatible with Mendelian inheritance) the social parent alleles in all six loci or had at the most one mismatched allele, assuming that this mismatch could have originated through mutation or typing error. We considered that nestlings were EPO if their alleles mismatched with those of the social male at two or more loci (García-Vigón *et al.* 2008a, b). Only these cases involving the offspring and their putative parents were genotyped again to confirm the results (García-Vigón *et al.* 2008a, b). When we removed the locus with more typing errors the combined exclusion probability was still high (0.941). We found that 9.5% (31/327) of nestlings were EPO and 27.2% (25/92) of broods contained at least one EPO. The rest of the nestlings were WPO. Of these, 306 matched in all alleles whereas 21 nestlings presented a single mismatch attributed to typing errors or mutations combined. We did not find any case of intraspecific nest parasitism (neither social male nor female matched) or pseudoparasitism (nestlings that mismatched the social female allele at two or more loci but matched the social male) in the analysed sample.

Measuring Paternal Care

To estimate paternal care we used the number of male feeding trips/h (male feeding rate; Allen & Islam 2004). We used male feeding rate of nestlings instead of other measures of male parental care because is easily quantifiable and is the variable used in most analyses of the association between extra-pair paternity and paternal care in birds. Furthermore, comparative analyses of the association between extra-pair paternity and paternal care in birds have revealed a strong relationship for male feeding of offspring but nonsignificant relationships for other components of male parental care (Møller & Cuervo 2000). Feeding rate at each nest was measured in 1998, 1999 and 2000 by means of two video camera sessions, each lasting 2 h, when nestlings were 4–5 and 10–12 days old respectively. The average rates for these two periods were used in our analyses (see Moreno *et al.* 1999 for more details of the rationale). Cameras were placed 3–6 m from of the entrance of the nestboxes and observations were made at 0800–1000 hours and 1600–1800 hours allowing parental identification by their individual combination of colour rings.

Data Analyses

To analyse data we used ANCOVA models (general linear models, SPSS Inc, Chicago, IL, U.S.A.). We calculated repeatability (Lessells & Boag 1987) of paternity and feeding rate of the same male in different broods as an indirect way to establish the degree of nonindependence of data when the same pair was included several times in the analyses. Repeatability of percentage of EPO in the brood was not significant ($R \pm SE = 0.087 \pm 0.116$, $n_0 = 3.174$, $F_{25,57} = 1.302$, $P = 0.203$) but repeatability of male feeding rate was significant ($R \pm SE = 0.233 \pm 0.134$, $n_0 = 2.925$, $F_{25,48} = 1.888$, $P = 0.026$), so we used different methods to avoid pseudoreplication. First, we performed an initial exploratory analysis in which we included all data collected across the 3 years. We used male feeding rate as the dependent variable and the presence or absence of EPO in the brood as a fixed factor. Furthermore, to control for differences in brood size and therefore for differences in brood demands, we included the number of nestlings fed in the brood (brood size) as a covariate, after having checked for the linearity of the male feeding trips/brood size relationship with a linear regression ($r = 0.267$, $F = 6.233$, $P = 0.015$).We also controlled for the possible effect female feeding rate may have on male feeding rate. We included other variables that were known or suspected to affect male parental behaviour such as (1) age of the male estimated as the number of years that a male had bred in the colony before the study year, (2) male mating status (i.e. whether he was monogamous or polygamous), (3) female mating status (i.e. whether she was primary or secondary) and (4) clutch order (i.e. whether it was the first or second clutch in the season). Finally, we included the variable year (1998, 1999 and 2000) as a fixed factor because the year could introduce variation in environmental conditions such as food abundance for breeding spotless starlings that in turn could contribute to differences in parental feeding rates. Taking year as either a fixed or a random factor did not make any significant difference to the outcome of our analyses (results not shown). We selected a main effects model in which we also included the interaction between clutch order and female status after having verified that the other interactions were clearly not significant (all $P > 0.200$). Since male feeding rate had a significant repeatability, we used a bootstrap procedure to avoid pseudoreplication. We ran an identical ANCOVA model 200 times, each time randomly selecting, with replacement, one value for each male and calculated average F statistics across the replicates (Yezerinac *et al.* 1996). To check whether the obtained F statistics were significant or not, we used the probability calculator of the statistical package STASTISTICA 6.0 (StatSoft Inc., Tulsa, OK, U.S.A.). Presence of EPO and the interaction between clutch order and female status were close to significance ($F_{1,39} = 3.087$, $P = 0.087$ and $F_{1,39} = 3.340$, $P = 0.075$, respectively). The other variables included in the model were not significant (all $P > 0.100$). The effect of the interaction between clutch order and female status could be masking a possible effect of the presence of EPO in the brood on male feeding rate. In fact, some studies have already shown that polygamous males do not feed at the secondary nest but feed at the primary nest at the same rate as monogamous males (Arenas 2000; Veiga *et al.* 2002; Smith *et al.*1995). These tendencies were also confirmed in our study. Using repeated measure ANCOVAs with male feeding rate as the dependent variable and brood size as covariate, we found a significant reduction in male feeding rates between the first (*Mean* ± *SE* = 2.21 ± 1.78 feeding trips/h, range 0–6.33) and second clutches (0.98 ± 1.17 feeding trips/h, range 0–4.33; $F_{1,18} = 6.429$, $P = 0.021$). Considering first clutches only, there was a significant difference between the average feeding rate of monogamous males (2.59 ± 1.83 feeding trips/h, range 0–6.33) and polygamous males for all their nests (1.32 ± 1.79 feeding trips/h, range 0–5; $F_{1,38} = 4.317$, $P = 0.045$). Nevertheless, no difference was found in feeding rates between monogamous and polygamous males in their primary nests ($F_{1,33} = 0.845$, $P = 0.365$). Taking into account the differences obtained above in relation to male feeding rate, we excluded second clutches and secondary nests of polygamous males from our final analyses relating paternity and parental care, except for reporting the variation in male feeding rate between broods of the same pair and breeding season. We performed our ANCOVA analyses by pooling all years together and using the same protocol as in the exploratory analysis but with some modifications to compare male feeding rate between broods of cuckolded and non cuckolded males. Based on previous analyses, we excluded clutch order, female status and their interaction.

To test the possible effect of the percentage of EPO in the brood on male feeding rate we followed the same protocol as before but we excluded the variable presence or absence of EPO in the brood from the analyses and included the percentage of EPO as covariate. We also analysed the pattern of parentage in successive breeding attempts because, according to theoretical models, it is also important in determining whether males will benefit or not from providing paternity-dependent care (Yezerinac *et al.* 1996). Therefore, we measured individual male parentage in multiple nesting attempts with the same female between different breeding seasons and in a single breeding season to test whether differences in male feeding rates within or between seasons were associated with differences in parentage. We used partial correlation coefficients of the SPSS 11.5 statistical package (SPSS Inc., Chicago, IL, U.S.A.) controlling for brood size.

Ethical Note

We bled nestlings at about 1 week of age when they weighed 50 g or more, obtaining 50 µl of blood from the jugular vein. We noted no adverse effects, and their development was the same as in a control plot in which no blood was sampled. If the youngest nestling was lighter than 50 g, we postponed sampling to a later visit. Adults sang and defended territories a few minutes after being bled (100 µl also from the jugular vein) and we never observed nest desertion or reduction in food provisioning or incubation bouts afterwards. The birds never showed any visible reaction to the video cameras and they seemed not to be affected by their presence. Spotless starlings are accustomed to human presence, as they normally breed in buildings, and the study colony has been monitored since 1995 with a high nestbox occupation and good breeding success yearly. The nest manipulation, ringing, bleeding and video recording were undertaken under licence from the Spanish Ministry of the Environment (General Directorate for Nature Conservation) and the Regional Government of Madrid.

RESULTS

After controlling for other influential variables and using ANCOVA models, we obtained a significant relationship between presence–absence of EPO in the nest and male feeding rate in 41.5% (83/200) of the analyses performed following the bootstrapping procedure, but rather than reducing their feeding rate in relation to the presence of EPO in their nest males increased it ($F_{1,22}$ = 4.471, P = 0.046). In other words, feeding rate was higher in cuckolded (*Mean* ± *SD* = 3.30 ± 1.88 feeding trips/h, range 0–6.50) than in non cuckolded males (2.02 ± 1.63, range 0–5.25; $F_{1,36}$ = 4.534, P = 0.040; Fig.1). Other variables included in the model (brood size, year, male status, male age and female feeding rate) were not significant for male feeding rate to nestlings. Brood size was significant 14 times (0.070%; $F_{1,22}$ = 1.396, P = 0.250), year three times (0.015%; $F_{2,22}$ = 0.685, P = 0.514), male status 10 times (0.050%; $F_{1,22}$ = 1.365, P = 0.255), male age 14 times (0.070%; $F_{1,22}$ = 1.361, P = 0.256) and female feeding rate never (0%; $F_{1,22}$ = 0.335, P = 0.569). The analysis of male feeding rates in relation to the percentage of EPO showed similar tendencies. Males with EPO in their broods had relatively higher feeding rates than males with no or few EPO in their brood but in this case, results were marginally nonsignificant ($F_{1,22}$ = 4.094, P = 0.055). Other variables in the model had no significant effect on male feeding rate (all P > 0.050). Furthermore, the percentage of EPO was unrelated to male age and male status.

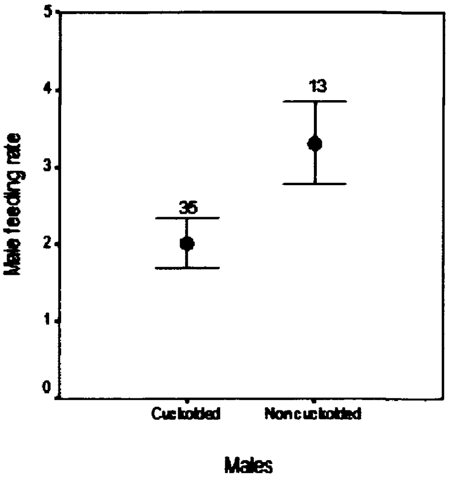

Figure 1. Feeding rates (feeding trips/h; Mean ± 1 SE) of male spotless starlings

in relation to the presence of extra-pair offspring (EPOs) in their broods. The number of sampled broods is given above the points.

A higher feeding rate of cuckolded males could be the compensating consequence of a reduced female feeding rate. To analyse this, we followed the same procedure as before, performing an ANCOVA analysis and a bootstrap procedure but using the female feeding rate instead of the male feeding rate. Female feeding effort was not associated with brood size ($F_{1,20}$ = 0.333, P = 0.570) and more promiscuous females did not feed broods differentially in relation to paternity ($F_{1,20}$ = 0.659, P = 0.427). We did not find any relationship between male feeding rate and paternity when we considered the successive breeding attempts of a male within a season. The share of paternity of the social father between two successive broods within a breeding season varied in seven (46.7%) of 15 pairs, but the direction of change between broods was not predictable. Social male paternity increased in three cases, decreased in four cases, and did not change in eight cases. In these pairs, after we controlled for differences in brood size, differences in male feeding rates between broods were not associated with differences in parentage (r_S = 0.001, N = 15, P = 0.998; Fig. 2).

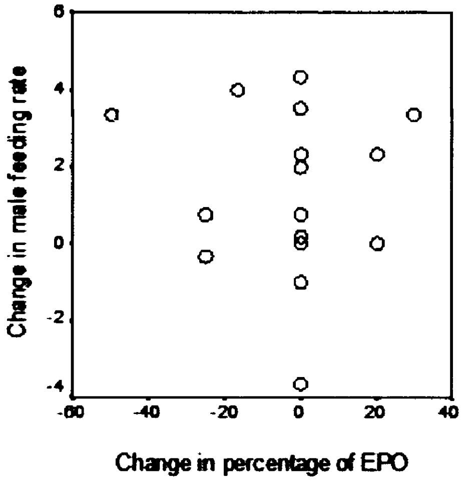

Figure 2. Paired changes in both feeding rate ('male feeding rate in first clutch' minus 'male feeding rate in second clutch') and paternity ('percentage of EPO in first clutch' minus 'percentage of EPO in second clutch') between broods within a breeding season.

For three of eight pairs with data for more than one breeding season the percentage of EPO in their broods varied between years. Differences in parentage between years did not imply differences in male feeding rates for any of these pairs (r_S = 0.014; N = 8, P = 0.794).

DISCUSSION

As expected, we did not find any evidence of a reduction in male feeding rate in relation to levels of EPO in broods but, surprisingly, cuckolded males provisioned nestlings more than non cuckolded males, a result rarely found in the literature (but see Hoi-Leitner *et al.* 1999). Within pairs, differences in male feeding rates for broods within and between seasons were not associated with differences in male paternity but we cannot discard here an effect of the small sample sizes analysed. The relationship between paternity and paternal care may be extraordinarily complex (Whittingham *et al.* 1992; Westneat & Sherman 1993; Ríos-Cardenas & Webster 2005) and it may depend on different factors that obscure correlational analyses (Kempenaers & Sheldon 1997). We can interpret our results from different perspectives and hypotheses. Simply and as predicted, the spotless starling is a species that apparently does not meet some of the conditions of theoretical models for parental care/paternity adjustment (Whittingham *et al.* 1992; Westneat & Sherman 1993). First, we found that levels of EPO did not vary between breeding attempts or with the age of the male in a predictable manner, contrary to studies of some other bird species (e.g. Morton *et al.* 1990; Wagner *et al.* 1996; Møller & Ninni 1998). Second, little empirical evidence is available for social males discriminating between sired and unrelated offspring within a brood. Furthermore, we also find it unlikely that male spotless starlings could judge their share of paternity using associative cues (Westneat *et al.* 1995). Some such cues could be related to social female behaviour during the breeding period such as frequency of extra-pair copulations (EPCs; Møller 1988; Ewen & Armstrong 2000), female periods of absence during egg laying (Sheldon *et al.* 1997) or mate-guarding efficiency (Davies & Hatchwell 1992; Whittingham & Dunn 1998). In the spotless starling, however, despite the rate of EPO found (Cordero *et al.* 2003; García-Vigón *et al.* 2008a, b), female EPCs must be a surreptitious behaviour. In fact, it has been rarely detected (Deceunink *et al.* 1997; J.P. Veiga & V. Polo, unpublished data). Furthermore, mate guarding seems to be far from efficient in this species (Deceunink *et al.* 1997) and it seems unlikely that males could use mate guarding as a cue for estimating paternity. A third condition of theoretical models for parental care/paternity adjustment states that benefits should offset the costs of reducing paternal care. The benefits for males reducing their feeding rate in relation to levels of EPO in the brood are strongly dependent on the mating system. In the case of polygynous bird species, in which extensive paternal care is less common than in monogamous species (Silver *et al.* 1985; Veiga *et al.* 2002), these benefits may consist of increasing the opportunities to engage in other activities that may increase fitness (Clutton- Brock 1991). In this case, we found no differences in feeding rates between monogamous and polygynous males when paired with primary females indicating that a reduction in parental care at the primary nest is not necessary for the acquisition of additional mates. Furthermore, Moreno *et al.* (1999) showed that the intensity of paternal care positively affected reproductive performance per breeding attempt, although this pattern was not consistent when they considered all nestlings raised by a male during the breeding season. This suggests that there is not a severe cost in terms of reduced survival of young when males reduce their paternal care as females can compensate for this cost. Furthermore, and contrary to all our expectation for a lack of relationship between parental care and levels of EPO, cuckolded males fed nestlings more than non cuckolded ones. We can link this result to various hypotheses. According to Hoi-Leitner *et al.* (1999), the high feeding rate of cuckolded males could be associated with high territory quality (food abundance) or female quality. These authors showed that, in high-quality territories, males had a lower feeding rate, probably because high abundance of food might facilitate more efficient food exploitation. Furthermore, in the framework of the constrained female hypothesis (Gowaty 1996), females in good environments or high-quality females are able to resist males, efforts to control mating better than females in poor environments or low-quality females, and, therefore, they have fewer EPO nestlings in their nest.

This could explain the pattern we and found could indicate that, contrary to previous studies, females could be the sex that controls EPCs in this species. Another possible interpretation of our findings could also be related to female and or male quality. If females compete to mate with the most attractive males, those females in poorer condition should be more likely to pair with unattractive males. These females could also be worse feeders and more likely to engage in EPCs with other males. Their male mates, in turn, would provision nestlings more in an attempt to compensate for being less attractive and for their mate's negligence, leading to a positive relationship between levels of EPOs and paternal care (Kempenaers & Sheldon 1997). However, this hypothesis, at least in respect to females, is unlikely to explain our findings as we found no differences in maternal provisioning between promiscuous and non promiscuous females. Alternatively, an increase in male parental care could be favoured if it acted to secure a mate for the next breeding season (Møller & Thornhill 1998; Seki *et al.* 2007). This may occur more often in relatively long-lived species with breeding site fidelity (Wagner 1992), characteristics that are also inherent to the spotless starling. In this case, provisioning by females does not need to be lower in broods of cuckolded males compared to broods of non cuckolded males, as occurred in our study, and the increase in male feeding rates does not compensate for the female's deficiencies in provisioning offspring but it should ensure that she pairs with him for future breeding attempts. This is because parental effort can have social consequences in the following years, as males with lower rates of provisioning may have a higher rate of divorce, and be less successful in obtaining future mates (also Wagner 1992; Dickinson 2003). An alternative hypothesis for our result is suggested from the offspring perspective. According to kin selection theory (Hamilton 1964), competition among nestlings increases if relatedness within broodmates is low. In birds, this has been corroborated by three studies on begging behaviour (Briskie *et al.* 1994; Boncoraglio & Saino 2008; Boncoraglio *et al.* 2009). In particular, the study on barn swallows, *Hirundo rustica*, showed that partially cross-fostered broods (i.e. the brood with lower relatedness among broodmates) begged more than natural, noncross-fostered broods, but the nestlings' morphological traits did not differ between the two groups. Begging behaviour includes visual and vocal signalling which increases in intensity according to hunger (Johnstone & Godfray 2002). If begging is costly in terms of, for example, energy requirements or other costs (e.g. McCarty 1996), a higher rate of begging in cross-fostered broods should be possible only if it increases provisioning by the attending parents. Here we found an increase in male feeding rate in the broods containing EPO, but we could not find the same tendency for female feeding rates. Nevertheless, this result was expected since feeding rate variance in females is much lower than in males. Females are more certain of their nestlings' parentage and are more willing to invest in them (Clutton-Brock 1991; Westneat & Sherman 1993). Although there is a study on begging behaviour in spotless starlings (Bulmer *et al.* 2008), this unfortunately does not deal with extra-pair paternity. Therefore, these results open a new and interesting line of research in which more information is badly needed. A higher feeding rate in cuckolded males than noncuckolded males was an unexpected result that deserves further investigation before generalization. Although the study included three breeding seasons in a large spotless starling colony, our level of significance and sample size were not very high. In any case, and because of the trend found, we are almost confident that males of facultatively polygynous species, such as the spotless starling, do not reduce their feeding rate when they lose paternity in the nest. This is probably because the search for extra-pair matings in males is of little relevance to individual fitness compared to a polygynous tactic of reproduction in a species in which EPO occurrence is unpredictable between breeding attempts or ages of the same male and reliable cues to assess paternity are unlikely.

ACKNOWLEDGEMENTS

We thank Marta Arenas, Sara Sánchez and Eduardo Mínguez for their help during the field work. We acknowledge José Miguel Aparicio for his inestimable help with Excel macros for the generation of samples to avoid pseudoreplication. We are also grateful to Vicente Polo for solving all our statistical doubts and for his comments on the manuscript. Finally, we thank Andrea Pilastro and two anonymous referees for their useful and detailed suggestions. During this work E.G-V. was supported by a predoctoral fellowship from the Ministerio de Educacio´n y Ciencia. This research was funded by project CGL-2005-05611-C02-01 and 02/BOS of the Ministerio de Educación y Ciencia and PCI08-0130 of the JCCM.

REFERENCES

Allen, J. & Islam, K. 2004. Gender difference in parental feeding effort of caerulean warblers at Big Oaks National Wildlife Refuge, Indiana. *Proceedings of the Indiana Academy of Science*, **113**: 162–165.

Arenas, M. 2000. *Inversión parental, estatus de emparejamiento e interferencia social en el estornino negro, Sturnus unicolor. Importancia del conflicto sexual en el desarrollo de estrategias de reproducción.* Tesina de licenciatura, Universidad Complutense de Madrid.

Birkhead, T. R. & Møller, A. P. 1992. *Sperm Competition in Birds. Evolutionary Causes and Consequences.* London: Academic Press.

Boncoraglio, G. & Saino, N. 2008. Barn swallow chicks beg more loudly when broodmates are unrelated. *Journal of Evolutionary Biology*, **21**: 256–262.

Boncoraglio, G., Saino, N. & Garamszegi, L. Z. 2009. Begging and cowbirds: brood parasites make host scream louder. *Behavioral Ecology*, **20**: 215–221.

Bouwman, K. M., Lessells, M. & Komdeur, J. 2005. Male reed buntings do not adjust parental effort in relation to extrapair paternity. *Behavioral Ecology*, **16**: 499–506.

Briskie, V. J., Naugler, C. T. & Leech, S. 1994. Begging intensity of nestling birds varies with sibling relatedness. *Proceedings* *of the Royal Society, Series B*, **258**: 73–78.

Bulmer, E., Celis, P. & Gil, D. 2008. Parent-absent begging: evidence for sibling honesty and cooperation in the spotless starling *(Sturnus unicolor)*. *Behavioral Ecology*, **19**: 279–284.

Chuang-Dobbs, H. C., Webster, M. S. & Holmes, R. T. 2001. Paternity and parental care in the black-throated blue warbler *Dendroica caerulescens*. *Animal Behaviour*, **62**: 83–92

Clutton-Brock, T. H. 1991. *The Evolution of Paternal Care*. Princeton: Princeton University Press.

Cordero, P. J., Veiga, J. P., Moreno, J. & Parkin, D. T. 2003. Extra-pair paternity in the nest of the facultatively polygynous spotless starling (*Sturnus unicolor*). *Behavioral Ecology and Sociobiology*, **54**: 1–6.

Davies, N. B. 1992. Dunnock *Behaviour and Social Evolution*. Oxford: Oxford University Press.

Davies, N. B. & Hatchwell, B. J. 1992. The value of male parental care and its influence on reproductive allocation by male and female dunnocks. *Journal of Animal Ecology*, **61**: 259–272.

Dawson, D. A., Hanotte, O., Greig, C., Stewart, I. R. K. & Burke, T. 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. *Molecular Ecology*, **9**: 1941–1943.

Deceunink, B., Peris, S. J., Calvo, J. M., Pascual, J. A. & De la Cruz-Cardiel, P. J. 1997. Mate guarding in the spotless starling (*Sturnus unicolor*): timing and duration pattern in intermediate breeding pairs. *Folia Zoologica*, **46**: 125–134.

Dickinson, J. L. 2003. Male share of provisioning is not influenced by actual or apparent loss of paternity in western bluebirds. *Behavioral Ecology*, **14**: 360–366.

Dixon, A., Ross, D., O'Malley, S. L. C. & Burke, T. 1994. Paternal investment inversely related to degree of extra-pair paternity in the reed bunting. *Nature*, **371**: 698–700.

Ewen, J. G. & Armstrong, D. P. 2000. Male provisioning is negatively correlated with attempted extrapair copulation frequency in the stitchbird (or hihi). *Animal Behaviour*, **60**: 429–433.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008a. Cross-amplified polymorphic microsatellites for the spotless starling *Sturnus unicolor*. *Ardeola*, **55**: 3–11.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008b. Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. *Animal Behaviour*, **76**: 345–353.

Gowaty, P. A. 1996. Battles of the sexes and origins of monogamy. In: *Partnerships in Birds* (Ed. by J. M. Black), pp. 21–52. Oxford: Oxford University Press.

Griffith, S. C., Owens, I. P. F. & Thuman, K. A. 2002. Extra-pair paternity in birds: a review of interspecific variation and adaptative function. *Molecular Ecology*, **11**: 2195–2212.

Hamilton, W. D. 1964. The genetical evolution of social behaviour. *Journal of Theoretical Biology*, **7**: 1–52.

Hoi-Leitner, M., Hoi, H., Romero-Pujante, M. & Valera, F. 1999. Female extra-pair behaviour and environmental quality in the serin (*Serinus serinus*): a test of the 'Constrained Female Hypothesis'. *Proceedings of the Royal Society of London, Series B*, **266**: 1021–1026.

Houston, A. I. 1995. Parental effort and paternity. *Animal Behaviour*, **50**: 1635–1644.

Johnstone, R. A. & Godfray, H. C. J. 2002. Models of begging as a signal of need. In: *The Evolution of Begging* (Ed. by J. Wright & M. L. Leonard), pp. 1–20. Dordrecht: Kluwer Academic.

Kempenaers, B. & Sheldon, B. C. 1997. Studying paternity and paternal care: pitfalls and problems. *Animal Behaviour*, **53**: 423–427.

Kempenaers, B., Lanctot, R. B. & Robertson, R. J. 1998. Certainty of paternity and paternal investment in eastern blue birds and tree swallows. *Animal Behaviour*, **55**: 845–860.

Kokko, H. 1999. Cuckoldry and the stability of biparental care. *Ecology Letters*, **4**: 247–255.

Lack, D. 1968. *Ecological Adaptations for Breeding in Birds*. London: Chapman &Hall.

Lessells, C. M. & Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. *Auk*, **104**: 116–121.

Lifjeld, J. T., Slagsvold, T. & Ellegren, H. 1998. Experimentally reduced paternity affects paternal effort and reproductive success in pied flycatchers. *Animal Behaviour*, **55**: 319–329.

McCarty, J. P. 1996. The energetic cost of begging in nestling passerines. *Auk*, **113**: 178–188.

MacDougall-Shackleton, E. A. & Robertson, R. J. 1998. Confidence of paternity and paternal care by eastern bluebirds. *Behavioral Ecology*, **9**: 201–205.

Maynard Smith, J. 1977. Parental investment: a prospective analysis. *Animal Behaviour*, **25**: 1–9.

Møller, A. P. 1988. Paternity and paternal care in the swallow, *Hirundo rustica*. *Animal Behaviour*, **36**: 996–1005.

Møller, A. P. & Cuervo, J. J. 2000. The evolution of paternity and paternal care in birds. *Behavioral Ecology*, **11**: 472–485.

Moller, A. P. & Ninni, P. 1998. Sperm competition and sexual selection: a metaanalysis of paternity studies of birds. *Behavioral Ecology and Sociobiology*, **43**: 345–358.

Møller, A. P. & Thornhill, R. 1998. Male parental care, differential parental investment by females and sexual selection. *Animal Behaviour*, **55**: 1507–1515.

Moreno, J., Veiga, J. P., Cordero, P. J. & Mínguez, E. 1999. Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **47**: 47–53.

Moreno, J., Veiga, J. P., Romasanta, M. & Sánchez, S. 2002. Effects of maternal quality and mating status on female reproductive success in the polygynous spotless starling. *Animal Behaviour*, **64**: 197–206.

Morton, E. S., Forman, L. & Braun, M. 1990. Extrapair fertilizations and the evolution of colonial breeding in purple martins. *Auk*, **107**: 275–283.

Neff, B. D. 2003. Decisions about parental care in response to perceived paternity. *Nature*, **422**: 716–719.

Peterson, K. A., Thusius, K. J., Whittingham, L. A. & Dunn, P. O. 2001. Allocation of male parental care in relation to paternity within and among broods of the common yellowthroat (*Geothlypis trichas*). *Ethology*, **107**: 573–586.

Richardson, D. S., Jury, F. L., Dawson, D. A., Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in *Sylviidae* species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**: 2226–2231.

Ríos-Cardenas, O. & Webster, M. S. 2005. Paternity and paternal effort in the pumpkinseed sunfish. *Behavioral Ecology*, **16**: 914–921.

Rubenstein, D. R. 2005. Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*. *Molecular Ecology Notes*, **5**: 739–744.

Seki, M., Wakano, J. Y. & Ihara, Y. 2007. A theoretical study on the evolution of male parental care and female multiple mating: effects of female mate choice and male care bias. *Journal of Theoretical Biology*, **247**: 281–296.

Sheldon, B. C. & Ellegren, H. 1998. Paternal effort related to experimentally manipulated paternity of male collared flycatchers. *Proceedings of the Royal Society of London, Series B*, **265**: 1737–1742.

Sheldon, B. C., Ra˜sa˜nen, K. & Dias, P. C. 1997. Certainty of paternity and paternal effort in the collared flycatcher. *Behavioral Ecology*, **8**: 421–428.

Silver, R., Andrews, H. & Ball, G. F. 1985. Parental care in an ecological perspective: a quantitative analysis of avian subfamilies. *American Zoologist*, **25**: 823–840.

Smith, H. G., Sandell, M. I. & Bruun, M. 1995. Paternal care in the European starling, *Sturnus* *vulgaris*: incubation. *Animal Behaviour*, **50**: 323–331.

Trivers, R. L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (Ed. by B. G. Campbell), pp. 139–179. Chicago: Aldine.

Veiga, J. P., Moreno, J., Arenas, M. & Sánchez, S. 2002. Reproductive consequences for males of paternal vs. territorial strategies in the polygynous spotless starling under variable ecological and social conditions. *Behavior*, **139**: 677–693.

Veiga, J. P., Viñuela, J., Cordero, P. J., Aparicio, J. M. & Polo, V. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. *Hormones and Behavior*, **46**: 47–53.

Wagner, R. H. 1992. Confidence of paternity and parental effort in razorbills. *Auk*, **109**: 556–562.

Wagner, R. H., Schug, M. D. & Morton, E. 1996. Confidence of paternity, actual paternity and parental effort by purple martins. *Animal Behaviour*, **52**: 123–132.

Weatherhead, R. M., Gibbs, H. L. & Boag, P. T. 1994. The cost of extra-pair fertilizations to female red-winged blackbirds. *Proceedings of the Royal Society of London , Series B*, **258**: 315–320.

Werren, J. H., Gross, M. R. & Shine, R. 1980. Paternity and the evolution of male parental care. *Journal of Theoretical Biology*, **82**: 619–631.

Westneat, D. F. 1995. Paternity and paternal behaviour in the red-winged blackbird, *Agelaius phoeniceus*. *Animal Behaviour*, **49**: 21–35.

Westneat, D. F. & Sherman, P. W. 1993. Parentage and the evolution of parental behaviour. *Behavioral Ecology*, **4**: 66–77.

Westneat, D. F. & Webster, M. S. 1994. Molecular analysis of kinship in birds: interesting questions and useful techniques. Experientia Supplementum (Basel). *Molecular Ecology and Evolution: Approaches and Applications*, **69**: 91–126

Westneat, D. F., Sherman, P. W. & Morton, M. L. 1990. The ecology and evolution of extra-pair copulations in birds. In: *Current Ornithology*. Vol. 7 (Ed.

by D. M. Power), pp. 331–369. New York: Plenum.

Westneat, D. F., Clark, A. B. & Rambo, K. C. 1995. Within-brood patterns of paternity and parental behaviour in red-winged blackbirds. *Behavioral Ecology and Sociobiology*, **37**: 349–356.

Whittingham, L. A. & Dunn, P. O. 1998. Male parental effort and paternity in a variable mating system. *Animal Behaviour*, **55**: 629–640.

Whittingham, L. A. & Dunn, P. O. 2001. Male parental care and paternity in birds. In: *Current Ornithology* (Ed. by V. Nolan, Jr & C. F. Thompson), pp. 257–298. New York: Plenum.

Whittingham, L. A. & Lifjeld, J. T. 1995. High paternal investment in unrelated young: extra-pair paternity and male parental care in house martins. *Behavioral Ecology and Sociobiology*, **37**: 103–108.

Whittingham, L. A., Taylor, P. D. & Robertson, R. J. 1992. Confidence of paternity and male parental care. *American Naturalist*, **139**: 1115–1125.

Whittingham, L. A., Dunn, P. O. & Robertson, R. J. 1993. Confidence of paternity and male parental care: an experimental study in tree swallows. *Animal Behaviour*, **46**: 139–147.

Whittingham, L. A., Dunn, P. O. & Clotfelter, E. D. 2003. Parental allocation of food to nestling tree swallows: the influence of nestling behaviour, sex and paternity. *Animal Behaviour*, **65**: 1203–1210.

Xia, X. 1992. Uncertainty of paternity can select against paternal care. *American Naturalist*, **139**: 1126–1129.

Yezerinac, S. M., Weatherhead, P. J. & Boag, P. T. 1996. Cuckoldry and lack of parentage-dependent paternal care in yellow warblers: a cost-benefit approach. *Animal Behaviour*, **52**: 821–832.

CAPÍTULO 4

Correlatos morfológicos y de edad del éxito de fertilización intra-pareja y promiscuo en el estornino negro

Elena García-Vigón, José Pablo Veiga & Pedro J. Cordero

RESUMEN

Tradicionalmente se han analizado las características fenotípicas y genéticas de los individuos reproductores con el fin de comprender la evolución y los mecanismos implicados en el éxito de las fertilizaciones intra y extra-pareja. Mientras que la mayor atención se ha puesto en las características de los machos, hay muy poca información acerca del papel que el fenotipo de la hembra tiene sobre las tácticas de emparejamiento y fertilización extra-pareja. Mediante análisis de parentesco usando marcadores microsatélite, nosotros testamos la importancia relativa que tienen los atributos fenotípicos (incluyendo la edad) de los machos territoriales y de las hembras de estornino negro sobre los patrones individuales del éxito de fertilización tanto intra-pareja como promiscuo. Mostramos que la longitud de las plumas del pecho es un correlato consistente de la edad tanto en machos como en hembras. Tanto la longitud de las plumas del pecho como el tamaño corporal de los machos estuvieron de manera independiente (ortogonal) y positiva asociados con el número de parejas sociales que estos atraían a sus nidos y por tanto con su éxito reproductor general, ya que los machos emparejados con más hembras simultáneamente no perdieron particularmente más paternidad que el resto. Ni la pérdida de paternidad del macho dentro de su propio nido, ni la ganancia de paternidad del macho en nidos ajenos, estuvo asociada con las características del macho pero si con las características de su pareja social. Los machos emparejados con hembras más maduras y con las plumas del pecho más largas perdieron menos paternidad en sus nidos y ganaron más paternidad en nidos ajenos que los machos emparejados con hembras más jóvenes y con las plumas del pecho más cortas. Las explicaciones de este patrón de apareamiento son dependientes del control que cada sexo tiene sobre la fertilización.

Palabras clave: edad, tamaño corporal, paternidad extra-pareja, microsatélites, ornamentos, *Sturnus unicolor*.

Morphometric and age correlates of promiscuous and pair fertilization success in the spotless starling

ABSTRACT

Phenotypic and genetic characteristics of mating individuals have been traditionally analysed in order to understand the evolution and mechanisms involved in intra and extra-pair fertilization success. Whereas most attention has been paid on male characteristics, there is very little information about the role of female phenotype on extra-pair mating tactics and extra-pair fertilization. Using microsatellite parentage assignment analyses, we test the relative importance that phenotypic attributes (including age) of territorial male and female spotless starlings have on patterns of individual promiscuous and pair fertilization success. We show that length of throat feathers is a consistent correlate of age in both males and females. Length of throat feathers and body size of males were independent (orthogonal) and positively associated with the number of social mates they attracted to their nests and thus with their overall breeding success, as more polygamous males were not particularly cuckolded. Neither paternity losses within own nests nor male paternity gains in alien nests were associated with male characteristics but both paternity loss within own nest and paternity gain in alien nest were associated with characteristics of the female partner. Males mated to older females with longer throat feathers were less cuckolded and obtained more extra pair fertilizations in alien nests than males mated to younger females with shorter ornaments. Explanations of this mating pattern are contingent on the control that each sex has on fertilization.

Keywords: age, body size, extra-pair paternity, microsatellites, ornaments, *Sturnus unicolor*

INTRODUCTION

Since molecular techniques have became an accessible tool for ecologists, many studies have been addressed to investigate the phenotypic correlates of males that are cuckolded and of those that gain additional paternity by fertilizing females other than their social partners. It is now clear that male characteristics may predict patterns of extra-pair paternity in birds although the morphological or behavioural correlates of extra-pair fertilization success vary considerably among species (Gowaty and Bridges 1991; Weatherhead & Boag 1995; Hasselquist *et al.* 1996; Sundberg & Dixon 1996; Kempenaers *et al.* 1997; Otter *et al.* 1998; Dunn & Cockburn 1999; Forstmeier *et al.* 2002).

A factor that is pervasively present in the theory about extra-pair fertilization is male quality. Many authors have championed the idea that selection favours females seeking for good genes so that females mated with poor or average males would enhance their fitness by getting additional matings with better males (Trivers 1972; Zahavi 1975; Møller 1988; Hamilton 1990; Westneat *et al.* 1990). However, it is crucial that females developing this strategy are able to "detect" such good genes using one or several unequivocal cues that are consistently present on their bearers. Age is a potential indicator of overall genetic quality as older individuals have demonstrated their ability to cope with the variety of factors that compromise survival (Trivers 1972; Halliday 1983; Manning 1985; Kokko 1998). Age is however difficult to evaluate by other individuals unless there are morphological traits that covary with it. In birds, some distinctive structures and colourations, or the degree of development of specialised feathers have been shown to be age dependent rather than condition dependent, suggesting that they may work as honest indicators of age (Sundberg & Larson 1994; Komdeur *et al.* 2005; Nicolaus *et al.* 2007). Also, a number of studies have found positive correlation between male age and fertilization success (Weatherhead & Boag 1995; Sundberg & Dixon 1996; Kempenaers *et al.* 1997; Richardson & Burke 1999; Schmoll *et al.* 2007; Laskemoen *et al.* 2008).

Nevertheless, ornaments of many different kinds are the traits that have been frequently viewed as clear evidences of the individual quality because the costs of their production and/or maintenance guarantee that their degree of development is unambiguously associated with some aspect of their owner's quality (Andersson 1994). Many empirical studies have succeeded in finding such correlations (e.g., Veiga & Puerta 1996; Sheldon *et al.* 1997; Keyser & Hill 2000; Siefferman & Hill 2003; Van Dongen & Mulder 2007; Bitton *et al.* 2008) and others have demonstrated that ornament development in males is a good predictor of intra or extra-pair fertilization success (Sundberg & Dixon 1996; Yezerinac & Weatherhead 1997; Møller *et al.* 1998; Johnsen *et al.* 2001; Bitton *et al.* 2007).

In contrast with this depicted scenario, it is surprising that there is practically no information about the role that the female phenotype has in extra-pair mating tactics and extra-pair fertilization (but see Wagner *et al.* 1996; Bowman & Komdeur 2005). This is even odder as both field and laboratory studies have demonstrated that whether an egg is fertilized by the sperm of the social male or by that of an extra-pair male is commonly under the female control (Lifjeld & Robertson 1992; Birkhead & Møller 1993). If mating is costly, it is not trivial to argue that females may develop condition dependent mating tactics in order to optimise their cost-benefits balance. Females of many species show traits that should be defined as ornaments, although most often these are less developed than those of males (e.g. plumage coloration in many birds: Johnson 1988; Muma & Weatherhead 1989; Stutchbury 1994). Such morphological traits may merely represent genetic correlates of male structures that have no functional value in females, as it has been stated for the genetic correlation hypothesis (Lande 1980; Lande & Arnold 1985; Hill 1993; Cuervo *et al.* 1996). However, female ornaments may be also the result of selection for their expression (Darwin 1981) and they can work as indicators of age or condition in the same way that they do in males. In fact, there are many recent studies in birds, both experiments and reviews, which show a relationship between the female phenotype and their quality (e.g.

Jones & Hunter 1993; Amundsen *et al.*1997; Amundsen 2000; Romero-Pujante *et al.* 2002; Jawor *et al.* 2004; Kraaijeveld *et al.* 2004; Griggio *et al.* 2005; Cornwallis & Birkhead 2007; Kraaijeveld *et al.* 2007; Morales *et al.* 2007). Thus, we suggest that a significant part of the variance observed in the patterns of intra and extra-pair paternity in birds may be caused by female strategies that are contingent on phenotype.

The spotless starling (*Sturnus unicolor*) is a facultatively polygynous passerine in which the proportion of nestlings originated from extra-pair fertilizations is comparatively high (about 15% of the offspring is extra-pair offspring (EPO) (Cordero *et al.* 2003; García-Vigón *et al.* 2008b)). We have previously shown that several male attributes can explain part of the variance in intra-pair fertilization success of the territorial males in this species (Cordero *et al.* 2003). However, we have also demonstrated that the experimental manipulation of female testosterone levels had a significant effect on the proportion of EPO found in broods showing that elevated levels of testosterone reduce the percentage of EPO in the brood (García-Vigón *et al.* 2008a). This same contribution also showed that female age was negatively associated with the proportion of EPO in their broods. Therefore, in this paper we are interested in the relative importance that the phenotypic attributes (including age) of both male and female spotless starlings may have on patterns of extra-pair paternity.

METHODS

Field procedures

We studied a colony at Collado Villalba (Madrid, Spain) comprising a plot of 40 nestboxes of 1.3 ha. The area is a pastureland with trees. A study of this colony has been underway since 1995. The spotless starling is a midsized, facultatively polygynous passerine. Depending on the year, 25%-45% of males that acquire a breeding territory mate with two or more females in our population, the remaining males acquire a single mate (Veiga *et al.* 2001, 2002). The laying season extends from mid-April to mid-June and most females lay two clutches per breeding season. (Mean = 4.9

eggs per clutch). Both male and females defend nests against competitors and intrasexual competition for nest sites is heavy in both sexes.

This study includes information gathered in 1998, 1999, 2000 and 2001. A proportion of breeders had already been marked in previous years and was individually recognized. Unmarked males were in most instances captured before egg laying using automatic nest traps, but some of them could only be trapped when they fed young. To identify the social male that controlled a nestbox we observed nestboxes during prelaying stages or, if a male fed nestlings, we trapped him or identified his rings on nestbox video recordings. The identity of social females breeding at each nestbox was determined in most cases by capturing them when they had nestlings 4-6 days old. Standard morphometric measurements (body mass, tarsus length, bill length, bill height, wing length and length of the outermost wing primary) and a blood sample were taken from captured individuals. We ringed all adults that were not yet marked with an aluminium ring and a combination of plastic colour rings to allow individual identification. Nestlings were ringed with aluminium rings when they were 5-6 days old and then bled. Blood was kept in the field in absolute ethanol and then stored at -40°C, until we used it to extract DNA.

In the spotless starling both sexes possess elongated throat feathers that are displayed during courtship and territorial exhibitions. The sexual dimorphism of this character is even higher than in the closely related European starling in which it is believe to be involved in sexual selection (Cuthill *et al.* 1999). Thus, we agree with authors that have suggested that throat feathers of starlings represent an ornamental trait (Aparicio *et al.* 2001; Komdeur *et al.* 2005). The mean length of three throat feathers pulled at random and measured with a digital calliper is designated in this study as Ornament length.

Breeding experience was measured as the number of years in which an individual bred in the study colony. We assumed that breeding individuals, which were not marked when they were trapped, had not previously bred and assigned them a zero value for this variable. This variable is closely related to age in females as 85% of the females of known age bred the year after their birth. The correlation is slightly smaller in males because males breed the second year or later after their birth (Cordero *et al.* 2001; V. Polo and J.P. Veiga unpubl. data). For simplicity, we refer to this variable as Age. As the monitoring of the studied population started in 1995, the variability of the age of focal males was considerable, with individuals breeding by first time in 2001 and other ones having a four years experience in 1998 when this study started.

Genetic Analyses

To estimate total male reproductive success we carried out microsatellite genotyping on families whose social parentage have been preliminarily determined by continuous observation of individually ringed birds in the colony. (For more details about the genotyping method see García-Vigón *et al* 2008a). We determined the true genetic relationships of 404 nestlings of 48 breeding females and 36 putative social males.

To extract and purify genomic DNA from the blood samples, we used NucleoSpin ® Tissue Kit (Macherey-Nagel, Duren, Germany). To determine parentage we used the following cross-amplifying microsatellite primers: Pca-7 (Dawson *et al.* 2000), Ase-18 (Richardson *et al.* 2000), and SS2.32, SS1.6, SS1.106, SS.2.106 (Rubenstein 2005). These microsatellites were polymorphic for spotless starling with an average of 8 alleles per locus (range: 4-12) and had a combined exclusion probability of 0.973 (García-Vigón *et al.* 2008a). Their suitability for paternity analyses has been validated in previous studies (e.g. García-Vigón *et al.* 2008a, b, 2009).

We assessed the genetic parentage and therefore the loss of paternity of territorial males by comparing nestling genotypes to those of the attending female and then to the territorial male at six loci. We considered that nestlings were within-pair offspring (WPO) if their alleles matched (i.e. they possessed alleles of the size of each parent and segregation was compatible with Mendelian inheritance) the social parents alleles in all six loci or had at the most one mismatched allele, assuming that this mismatch could be originated through mutation or typing error. We considered that nestlings were EPO if their alleles mismatched with those of the social male at two or more loci (García-Vigón *et al.* 2008a). We consider that nestlings were intraspecific-nest parasite offspring when their alleles mismatches with both their social male and social female after discarding samples assignment errors. We consider that nestlings were pseudoparasite offspring if their alleles mismatches with those of their social female but matched those of their social male. Only these cases with two or more mismatches, involving the offspring and their putative parents, were genotyped again to confirm the obtained results (García-Vigón *et al.* 2008 a, b). When we removed the locus with more typing errors the combined exclusion probability still was very high (0.941). We found that 10.4% (42/404) of studied nestlings were EPO and 30.7% (35/114) of studied broods contained at least one EPO. We also found 3 parasite nestlings and 3 pseudoparasite nestlings. The rest of nestlings (356) were WPO. Of these, 0.09% presented a single mismatch attributed to typing errors or mutations combined.

We determined male extra-pair gains of paternity in alien nests by using CERVUS, version 2.0 (Marshall *et al.* 1998) a likelihood-based computer program to assign the most probable genetic paternity of an EPO nestling among a set of different putative males.

We run the program with one simulation for each study year. Simulation parameters for 1998 were 95.4% of loci typed and 33 candidate parents of which 82% were sampled. Simulation parameters were 99.2% of loci typed and 44 candidate parents of which 86% were sampled for 1999; 98.9 % of loci typed and 39 candidate parents of which 87% were sampled for 2000, and 99.4% of loci typed and 34 candidate parents of which 71% were sampled for 2001. We run all simulations for 10000 cycles assuming an error rate of 1%, the default value in CERVUS. We estimated the number of candidate parents increasing the field data (number of territorial and observed floating males in the colony) by approximately 20% to account for parentage by not sampled individuals (Berg 2005).

Data analyses

Our general design consisted of testing the effect that age and morphometric variables of males and females had on several dependent variables estimators of males reproductive success. We considered the following dependent variables: Number of mates (the number of females mated with a male in a breeding season), Paternity loss within nest (the arcsine square root of the percentage of EPO in the nest of the social mate/s) and Paternity gain outside nest. This last variable was defined as the presence or absence of extra-pair young sired by a male in the nests of females other than the social mates. We chose this instead of a continuous variable because only in two cases males obtained more than one EPO. Some males (and females) included in the analyses bred in two or more rarely in three or four breeding seasons. Thus, to circumvent pseudoreplication, morphometric data and age corresponding to the same male collected in more than one year were averaged to obtain a single value per individual. Morphometric data and age of different females mated with a male in a season were also averaged and the resulting values were averaged again to obtain figures that characterize the "average mate" of each focal male. We used both principal component analysis (PCA) and regression analyses (see below) based on 36 males for which we were able to establish the genetic relationships with their nestling.

To reduce the probability of type I errors and simplify the pool of morphometric variables measured to a reduced set of intercorrelated variables, we performed a PCA in each of the two sexes (Frey and Pimentel 1978; Tabacknick and Fidell 2007). We included Age because we were interested in discriminating the morphological variables that exhibit a consistent pattern of variation throughout the lifetime of an individual. We extracted components whose eigenvalues were greater than 1. The resulting matrix was rotated by the Varimax procedure. The coordinates of the individuals in each of the extracted components were used as independent variables to test the influence of male and female attributes on the dependent variables.

To test the effect that components have on the Number of mates we used multiple regression analysis. The actual number of females attracted by a male is hard to know, so that we defined mated females as those females that started a clutch throughout a breeding season. To test the effect that the resulting components could have on Paternity loss within nest we also used multiple regression analysis. The mating status of the male was a potential correlate of extra-pair paternity because we had previously found that polygynous males lose paternity at least in some years (Cordero *et al.* 2003). Thus, we included Number of mates as an additional variable in the analysis. The effect of components on Paternity gain outside nest, a dichotomous variable, was tested by means of a logistic regression. Because some of the males did not breed in each of the four years considered in this study, we included the Number of years breeding as an additional variable in the analysis to control its effect on the dependent variable. All analyses were conducted using the SPSS statistical package (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Principal components of morphology

The factorial analysis extracted three principal components for the male variables that explain 68.4% of the variance. The first component gathers high positive loadings for wing length and length of the outermost wing primary (Table 1). This variation axis represents the degree of development of flight feathers (hereafter Flight feathers). However, Ornament length, a feather trait, has smaller values in this component. Age has negative loadings on this component suggesting that the development of flight feathers may decrease with age, although the value is very small to consider that this effect is relevant. The second component has the highest loadings for body mass but also includes relatively high values for tarsus length, bill length and bill size. This axis represents the general size of male body (hereafter Body size). The significance of age in this variation axis is practically null.

This component also evidences that Ornament length is entirely independent of body size. The third component associates positively Ornament length with Age indicating unambiguously that the throat feathers increase as the male becomes older (hereafter Ornament-Age).

For the average females mated with males, the general pattern is similar (Table 1). The first component also gathers high positive loadings for wing length and outermost primary length, but in this sex also the body mass has a major significance in this axis. It is worth noting that body mass in females is rather independent of the length of those structures that reflect body size suggesting that this trait is more dependent on environmental factors than it is in males. Thus, the association of body mass and flight feather length may be indicating that body condition may favour feather development during moult. We term this first component as Body mass-Flight feathers. The second component is equivalent to the third component of males, with highest loadings for Ornament length and Age. Bill height also has relatively high positive values on this component but the significance of its association with the other two variables that define this component is not clear. The third component associates tarsus length and bill length suggesting that it is equivalent to the second component of males and mostly reflects general size of the body.

Male attributes, number of mates, female attributes and fertilization success

Two male components showed significant association with the Number of mates: Body size and Ornament-Age (Table 2). Beta coefficients are positive indicating that larger males and, independently, those being older and with longer ornaments succeeded in mating with a greater number of females. The same analysis also showed that female components are unrelated to the Number of mates acquired by males. This result suggests that the characteristics of the females mated with the same male do not vary in accordance with male acquisition of more mates and so these females are not particularly younger or smaller.

None of male components was related to Paternity loss within nest (Table 3). Rather, this variable was affected by some female attributes as evidenced by its association with the female Ornament-Age component. Beta coefficient is in this case negative indicating that older females with longer ornaments had a smaller proportion of extra-pair young in their broods (Table 3).

As in the case of the proportion of Paternity losses within nests, Paternity gain outside nest was not dependent on male attributes but on their female Ornament-Age component (Table 4). However, the relation is now positive indicating that males mated with more ornamented-older females also sired more young in the nests of other females.

Table 1. Principal Component Analyses (PCA) of morphometric variables.

	Male Components			Female Components		
	1	2	3	1	2	3
Explained variance	33.76	18.47	16.17	27.82	23.34	18.19
Variables						
Wing length	0.940	0.189	-0.074	0.875	-0.111	0.124
Wing feather length	0.946	0.111	0.014	0.833	0.105	0.109
Tarsus length	0.156	0.522	-0.332	-0.044	0.152	0.785
Bill length	0.296	0.687	-0.075	0.141	-0.108	0.841
Bill height	-0.196	0.658	0.025	-0.019	0.625	0.245
Body mass	0.299	0.802	0.231	0.830	0.053	-0.123
Ornament length	0.230	-0.079	0.768	0.082	0.844	-0.252
Age	-0.354	0.101	0.790	0.002	0.847	0.040

Table 2. Multiple regression of the Number of mates on male and female components (C).

	Beta	T	P
Constant		17.06	<0.001
Male C1 (Flight feathers)	-0.18.	-1.2	0.24
Male C 2 (Body size)	0.39	2.45	0.021
Male C 3 (Ornament-Age)	0.33	2.09	0.045
Female C 1 (Body mass-Flight feathers)	0.12	0.79	0.43
Female C 2 (Ornament-Age)	-0.06	-0.4	0.69
Female C 3 (Body size)	0.005	0.03	0.97

See text and Table 1 for the characterization of the components.

Table 3. Multiple regression of Paternity loss within nest (the percentage of extra-pair offspring within the broods of the social mates) on male and female components.

	Beta	T	P
Constant	2.74		0.01
Male C 1 (Flight feathers)	-0.04	-0.26	0.80
Male C 2 (Body size)	-0.22	-1.22	0.23
Male C 3 (Ornament-Age)	0.20	1.14	0.26
Female C 1 (Body mass-Flight feathers)	0.10	0.64	0.53
Female C 2 (Ornament-Age)	-0.44	-2.8	0.009
Female C 3 (Body size)	-0.18	-1.1	0.28
Number of mates	-0.10	-0.56	0.60

Number of mates is included in the model to control the eventual effect of the male's mating status on the dependent variable.

Table 4. Logistic regression of Paternity gain outside nest (the presence or absence of extra-pair offspring sired by a male in nests of females other than their social mates) on male and female components.

	B	Wald	P
Constant	-16.37	4.09	0.43
Male C 1 (Flight feathers)	1.01	0.94	0.33
Male C 2 (Body size)	0.88	1.31	0.25
Male C 3 (Ornament-Age)	-0.37	0.10	0.75
Female C 1 (Body mass-Flight feathers)	-2.33	2.52	0.11
Female C 2 (Ornament-Age)	3.0	4.45	0.035
Female C 3 (Body size)	-0.31	0.11	0.74
Number of years breeding	4.88	3.89	0.049

Number of years breeding is included in the model to control the eventual effect of the time a male was breeding in the study colony on the dependent variable.

DISCUSSION

Ornaments: age or anything else?

Our PCA revealed that Ornament length was more associated to Age than to any morphometric variable. This result, with some differences, was recorded in both sexes evidencing a growth of the throat feathers that occurs throughout lifetime. In fact, lifetime growth monitoring of throat feathers in more than a hundred males and females of known age showed that ornaments invariably grows with age (J.P. Veiga and V. Polo unpubl. data). Furthermore, we sustain that ornament length may be used by an individual to make an accurate estimate of the age of opposite sex. In any case, we cannot disregard that the length or the ornaments is also correlated with other aspects of condition. It has been discussed in the European starlings whether ornaments are indicators of age or quality and it has been proposed that due to the statistical inseparability of ornamentation and age it is difficult in correlative approaches to establish whether ornaments may signal aspects of quality beyond those attributable to age (Komdeur *et al.* 2005). This same limitation is faced in our approach. However, in a previous study, Aparicio *et al.* (2001) found a positive relationship between ornament length and heterozygosity in male spotless starlings, though sample size was relatively small. More heterozygous individuals could also attain better survival because heterozygosity is closely related with the ability to cope with infections, parasites and with an enhanced function of enzymatic systems (Westerdahl *et al.* 2005; Ortego *et al.* 2007).

In any case, the consistent pattern of throat feather growing with age determines that any additional quality aspect that could be eventually signalled by ornaments should be shown through the residuals of Ornament length on Age. However, if a female or a male evaluate cryptic deviations of ornament length irrespective of age, they would probably use alternative quality traits. For example, in European starlings (and probably in the spotless starlings) song complexity vary with age (Eens *et al.* 1991; Absil *et al.* 2003), so that it is possible that females (and also males) could perceive the congruence between song complexity and ornaments size to make an integrative evaluation of age vs. quality. However, this argument is speculative and further information on this topic is badly needed. Further, an additional PCA performed including the residuals of Ornament length on Age showed a weak association of this new variable with any other trait either in males or females (results not shown) suggesting that deviation of Ornament length from the general trend with Age is probably erratic.

Thus, we suggest that ornament length is primarily a good estimator of age for both male and female spotless starlings in which age is crucial to assess individual quality. Old males attract more females than young males and this output is in part caused by the greater ability of older males to defend more nesting sites than younger ones. Older males not only have proved good viability that may be heritable (Kokko 1997), but also superior fighting abilities that may be a guarantee to females of a more efficacious defence of them or their young. A similar pattern of female preferences for older males has been shown in several species (Weatherhead 1984; Alatalo *et al.* 1986). On the other side, older females, aside of proving good viability, have a superior fecundity and reproductive performance (see also Komdeur *et al.* 2005). Thus, we think the Ornament-Age axis makes sense as a measurable integrative trait embracing throat feather length and age that in turn may be traducing honest signalling and a more realistic picture of quality that individuals may perceive of opposite sex. In any case, we cannot disentangle the possibility of a common underlying genetic basis that could be affecting different traits associated with individual quality like survival and ornament length.

Male attributes and number of mates

Our results show that two of the three extracted components of the male attributes had a significant and independent effect on the Number of mates. The second component, that represents a body size variation axis, had the strongest effect. This is not surprising as we previously found that the number of females attracted by a male is primarily determined by the number of nests he is able to defend (Veiga *et al.* 2001, 2006) and the association between male size and resource holding potential has been repeatedly reported (Lozano 1994; Briffa & Sneddon 2007). However, even some males that only acquired a nest site were able to attract more than one female. We have defined mated females as those that laid a clutch so that this excludes females that were not able to have a nest for her exclusive use. Some mated females could lay parasitically or breed communally in the nests of the other females mated with the same male (J.P. Veiga unpubl. data), while others probably remained as non-layers suggesting that, the actual number of females mated with a male is very difficult to assess in this species. Thus, we cannot conclude that bigger males acquired more females but bigger males controlled a number of nests in which more clutches were started across a breeding season. In any case, bigger males had an apparent greater reproductive success.

The second variable explaining the number of females mated by a male is Ornament-Age. This effect must be considered independently of size as both components are orthogonal (Frey & Pimentel 1978; Tabachnick & Fidell 2007). These results suggest that superimposed to the size effect, more ornamented-older males are able to attract more females. However, our approach does not enable us to determine the relative importance of age and ornaments on the number of females mated by a male. The strong association between Ornament length and Age suggests that the environmental effects on the Ornament-Age axis are probably small, so females could hardly evaluate current condition of potential mates from this trait only. Other studies have shown a consistent correlation between ornament development and male condition, which could explain the preference of ornamented males by females (e.g. Trivers 1972; Zahavi 1975; Zuk *et al.* 1990; Andersson 1994; Johnstone 1995; Veiga & Puerta 1996; Cotton *et al.* 2004; Tarof 2005; Garvin *et al.* 2008). For the spotless starling, in which ornament length is a good and reliable predictor of age, we cannot reject the possibility that females can evaluate current physical condition of potential partners by watching other traits. Nonetheless, the growth of ornaments in males occurs mostly between the first and second years of age but after the third year no consistent pattern of growth was detected (V. Polo and J.P. Veiga unpubl. data), so that this trait could enable females to easily discriminate yearlings and/or two years old males from older males. Yearlings usually do not breed in our studied populations but they may in some occasions acquire a nest opportunistically and fertilize females. Young males however are always subordinated to older males so that it is risky for females to have a social male that cannot guarantee the defence of the nest and young against an older male taking over a territory. Incoming males may toss eggs or kill young to mate with the victimized female. This is a common behaviour in birds and it is documented in both the European starling and the spotless starling (reviewed by Veiga 2000).

In summary, ornaments predict mating success probably because more ornamented-older males could offer a better defence of the nest and young again conspecifics and perhaps predators. In addition, older males have demonstrated a survival ability that could be indicating a genetic value, but the relative importance that for females have genetic and immediate reproductive benefits have to be established in this species.

Extra-pair paternity: females set the rule

Our results indicate that male characteristics are not related to rates of cuckoldry suffered within own broods. Males mated with more females per breeding season across the four years of the study had similar success, in terms of percentage of sired offspring per brood, than males that mated with a smaller number of females. Most relevant for the aims of this study is that Ornament-Age and Paternity loss within nest are not correlated. Thus, more ornamented-older males are able to fertilize their social females at the same rate than less ornamented males irrespective of their mating status. Although the loss of paternity has been recognized as a major cost of being polygynous (Freeland *et al.* 1995; Hasselquist & Sherman 2001; Pilastro *et al.* 2002), our results suggest that more ornamented-older males, by mating with a higher number of females, increased their total reproductive success (e.g. Kempenaers *et al.* 1995; Smith & Vonschantz 1993; Westneat 1993).

The Ornament-Age axis of males did not affect Paternity gain outside nest. A prediction of the theory is that females searching for extra-pair mates should prefer males with bigger ornaments if these traits are a signal of genetic pre-eminence (e.g. Norris 1993;

Petrie 1994). As discussed above, large ornaments and age could be indicating an underlying genetic quality in the spotless starling although there is no consistent evidence on this respect. However, if we assume the role of "good genes", even "genetic champions" should exhibit short ornaments when they are young. So, it is possible that females do not look for long ornaments when they search for an extra-pair male. It is possible that other traits, or a combination of traits, are more precise indicators of the genetic heritage searched by females (Pomiankowski & Iwasa 1998; Andersson *et al.* 2002; Badyaev & Young 2004; Van Doorn & Weissing 2004 and references therein; Hegyi *et al.* 2007).

The female Ornament-Age was then the only variable, among those included in this study, showing a correlation with the fertilization success of their social mates. Males mated with more ornamented-older females were less cuckolded and they sired more EPO in other nests. Provided that males gaining paternity in alien nests are similar in age and morphology to non-promiscuous males, we cannot advance any selective advantage (in terms of male quality) for females having EPO. If fertilization is under the female control (e.g. Lifjeld & Robertson 1992; Birkhead & Møller 1993), the most parsimonious explanation of this result is that experienced females are more capable to avoid forced copulations or to select sperm (Wagner *et al.* 1996). However, it is possible that juvenile females, that regularly mate with younger males (assortative mating by age is the rule in our population), are more prone to promiscuous copulations and we cannot rule out the eventual benefits of this multiple mating (e.g. increase of the genetic diversity and/or of the heterozigosity of their progeny; reviewed in Jennions & Petrie 2000; Foerster *et al.* 2003). On the other side, if a male has some control on fertilizations, it is possible that males mated to more ornamented- older females invest more in protecting paternity than if they mate with younger females. This is consistent with the fact that older female starlings represent a more valuable resource because they are more fertile and raise more young as occurs in many other species in which female breeding success improve with advancing age (reviewed in Forslund & Pärt 1995; Angelier *et al.* 2007).

The fact that males mated with more ornamented-older females showed the highest rates of paternity gains in alien nests is a bit surprising as one could argue that experienced females could possess superior skills to prevent the promiscuous behaviour of their mates. However, this assertion assumes that such male behaviour is costly to females, a proposal that it is not adequately demonstrated (but see Lubjuhn 2005). It is possible that expertise renders older females to be more prudent and more prone to remain near the nest during the fertile period while juveniles prefer to remain close to the male reducing his chances to copulate with other females. Although some evidence of these differences in the behaviour of juvenile and old female starlings have been recorded (J.P. Veiga unpubl. data), the argument clearly needs additional support. One possibility is that searching for extra-pair mates is more costly for older females, perhaps because of physiological constraints such as accumulated oxidative stress because of the aging process, which could result in a less appetence for copulation with extra-pair males. Alternatively, if more ornamented-older females seem to prevent or search for extra-pair copulations less than younger and with shorter ornaments females, it is possible that males mated with the former invest less time in mate guarding and more time in trying to copulate with other females.

In sum, this study shows that ornament length is a consistent correlate of age in both males and females and probably does not give significant additional information on phenotypic or genetic features of their bearers. The size of the ornaments and the general size of males were independently associated with the number of social mates they attracted to their nests. This suggests that bigger males defend more nest sites and that more ornamented-older males may be preferred by females because of their greater quality (either phenotypic or genetic). The fertilization success of males either with their social mates or with other females is determined not by their own attributes but by the Ornament-Age of females with which they were mated. Males mated with more ornamented-older females were less cuckolded and had more EPO in other nests. Explanations of this pattern are contingent on the control that each sex has on fertilization.

ACKNOWLEDGEMENTS

During this work, E.G-V was supported by a predoctoral fellowship from the Ministerio de Ciencia y Tecnología. This research was funded by projects CGL2005-05611-C02- 01/BOS, CGL2005-05611-C02-02/BOS and CGL2008-02843. Authorization for field work was provided by the Comunidad de Madrid.

REFERENCES

Absil, P., Pinxten, R., Balthazart, J. & Eens, M. 2003. Effect of age and testosterone on autumnal neurogenesis in male European starlings (*Sturnus vulgaris*). *Behavioral Brain Research* **143**: 15-30.

Alatalo, R.V., Gustafsson, L. & Lundberg, A. 1986. Do females prefer older males in polygynous bird species?. *American Naturalist,* **127**: 241-245.

Amundsen, T. 2000. Why are female birds ornamented? *Trends in Ecology & Evolution,* **15**: 149-155.

Amundsen, T., Forsgren, E. & Hansen, L. T. T. 1997. On the function of female ornaments: male bluethroats prefer colourful females. *Proceedings of the Royal Society of London, Series B,* **264**:1579-1586.

Andersson, M. 1994. *Sexual selection.* Princeton: Princeton University Press.

Andersson, S., Pryke, S. R., Örnborg, J., Lawes, M. J. & Andersson, M. 2002. Multiple receivers, multiple ornaments, and a trade-off between agonistic and epigamic signalling in a widowbird. *American Naturalist,* **160**: 683-691.

Angelier, F., Moe, B., Weimerskirch, H. & Chastel, O. 2007. Age-specific reproductive success in a long-lived bird: do older parents resist stress better?. *Journal of Animal Ecology.* **76**:1181–1191.

Aparicio, J. M., Cordero, P. J. & Veiga, J. P. 2001. A test of the hypothesis of mate choice based on heterozygosity in the Spotless Starling. *Animal Behaviour,* **62:** 1001-1006.

Badyaev, A. V. & Young, R. L.. 2004. Complexity and integration in sexual ornamentation: an example with carotenoid and melanin plumage pigmentation. *Journal of Evolutionary Biology*, **17**: 1317-1327.

Berg, E. 2005. Parentage and reproductive success in the white-throated magpie-jay, *Calocitta formosa*, a cooperative breeder with female helpers. *Animal Behaviour*, **70**: 375-385.

Birkhead, T. & Møller, A. 1993. Female control of paternity. *Trends in Ecology & Evolution*, **8**: 100-104.

Bitton, P. P., O'Brien, E. L. & Dawson, R. D. 2007. Plumage brightness and age predict extrapair fertilization success of male tree swallows, *Tachycineta bicolor*. *Animal Behaviour*, **74**: 1777-1784.

Bitton, P. P., Dawson, R. D. & Ochs, C.L. 2008. Plumage characteristics, reproductive investment, and assortative mating in tree swallows *Tachycineta bicolor*. *Behavioral Ecology and Sociobiology*, **62**: 1543-1550.

Briffa, M. & Sneddon, L.U. 2007. Physiological constraints on contest behaviour. *Functional Ecology*, **21**: 627-637.

Cordero, P. J., Veiga, J. P., Moreno J. & Parkin, D.T. 2003. Extra-pair paternity in the facultatively polygynous Spotless Starling, *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **54**:1-6.

Cordero, P. J., Viñuela, J., Aparicio, J. M. & Veiga, J. P. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the Spotless Starlings. *Journal of Evolutionary Biology*, **14**: 829-834.

Cornwallis, C. K. & Birkhead, T. R. 2007. Experimental evidence that female ornamentation increases the acquisition of sperm and signal fecundity. *Proceedings of the Royal Society of London, Series B*, **274**: 583-590.

Cotton, S., Fowler, K. & Pomiankowski, A. 2004. Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis?. *Proceedings of the Royal Society* *of London, Series B*, **271**: 771-783.

Cuervo, J. J., de Lope, F. & Møller, A. P. 1996. The function of long tails in female barn swallows (*Hirundo rustica*): an experimental study. *Behavioral Ecology*, 7: 132-136.

Cuthill, I. C., Bennet, A. T. D., Partridge, J. C. & Maier, E. J. 1999. Plumage reflectance and the objetive assessment of avian sexual dichromatism. *American Naturalist,* **153**: 183-200.

Darwin, C. 1871. The Descent of Man, and Selection in Relation to Sex. John Murray, London, United Kingdom

Dawson, D. A., Hanotte, O., Greig, C., Stewart, I. R. K. & Burke, T. 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbirds families. *Molecular Ecology*, **9**: 1941-1944.

Dunn, P. O. & Cockburn, A. 1999. Extrapair mate choice and honest signaling in cooperatively breeding superb fairy-wrens. *Evolution*, **53**: 938-946.

Eens, M., Pinxten, R. & Verheyen, R. F. 1991. Male song as a cue for mate choice in the European starling. *Behaviour*, **116**: 210-238.

Foerster, K., Delhey, K. Johnsen, A. Lifjeld, J. T. & Kempenaers, B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature*, **425**: 714-717.

Forstlund, P. & Part, T. 1995. Age and reproduction in birds-hypotheses and test. *Trends in Ecology & Evolution,* **10**: 374-378.

Forstmeier, W., Kempenaers, B., Meyer, A. & Leisler, B. 2002. A novel song parameter correlates with extra-pair paternity and reflects male longevity. *Proceedings of the Royal Society of London, Series B,* **269**: 1479-1485.

Freeland, J. R., Hannon,S. J., Dobush, G. & Boag, P. T. 1995. Extra-pair paternity in willow ptarmigan broods: measuring costs of polygyny to males. *Behavioral Ecology and Sociobioly,* **36**: 349-355.

Frey, D. F., & Pimentel, R. A. 1978. Principal component analyses and factor analyses. In: *Quantitative Ethology* (Ed. by P.

W. Colgan), pp 219-246. New York: John Wiley & Sons.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008a. Cross-amplified polymorphic microsatellites for the Spotless Starling *Sturnus unicolor. Ardeola*, **55**: 3-11.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008b. Exogenous testosterone in female Spotless Starling reduces their rate of extrapair offspring. *Animal Behaviour,* **76**: 345-353.

García-Vigón, E., Veiga, J. P. & Cordero, P. J. 2009. Male feeding rate and extrapair paternity in the facultative polygynous Spotless Starling. *Animal Behaviour,* **78**: 1335-1341.

Garvin, J. C., Dunn,P. O., Whittingham, L. A., Steeber, D. A. & Hasselquist, D. 2008. Do male ornaments signal immunity in the common yellowthroat?. *Behavioral Ecology,* **19**: 54-60.

Gowaty, P. A. & Bridges, W. C. 1991. Behavioural, demographic, and environmental correlates of extrapair fertilizations in Eastern bluebirds, *Sialis sialis*. *Behavioral Ecology,* **2**: 339-350.

Griggio, M., Valera, F., Casas, A. & Pilastro, A. 2005. Males prefer ornamented females: a field experiment of male choice in the rock sparrow. *Animal Behaviour* , **69**: 1243-1250.

Halliday, T. R. 1983. The study of mate choice. In: *Mate Choice* (Ed. by P. Bateson), pp 3-32. Cambridge: Cambridge University Press.

Hamilton, W. D. 1990. Mate choice near or far. *American Zoologist,* **30**: 341-352.

Hasselquist, D. & Sherman, P. W. 2001. Social mating systems and extrapair fertilizations in passerine birds. *Behavioral Ecology,* **12**: 457-466.

Hasselquist, D., Bensch, S. & von Schantz, T. 1996. Correlation between male song repertoire, extra-pair paternity and offspring survival in the read great warbler. *Nature*, **381**: 229-231.

Hegyi, G., Szigeti, B., Török, J. & Eens, M. 2007. Melanin, carotenoid and structural plumage ornaments: information content and role in great tits *Parus major. Journal of Avian Biology,* **38**: 698-708.

Hill, G. E. 1993. Male mate choice and the evolution of female plumage coloration in the house finch. *Evolution*, **47**:1515-1525.

Jawor, J. M., Gray, N. S., Beall, A. & Breitwisch, R. 2004. Multiple ornaments correlate with aspects of condition and behaviour in female northern cardinals, *Cardinalis cardinalis*. *Animal Behaviour,* **67**: 875-882.

Jennions, M. D. & Petrie, M. 2000. Why females mate multiply? A review of the genetic benefits. *Biology Reviews,* **75**: 21-64.

Johnsen, A., Lifjeld, J., Andersson, S., Örnborg, J. & Amundsen, T. 2001. Male characteristics and fertilisation success in bluethroats. *Behaviour,* **138**: 1371-1390.

Johnson, K. 1988. Sesual selection in pinyon jays. II: male choice and female-female competition. *Animal Behaviour,* **36**: 1048-1053.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. *Biology Reviews,* **70**: 1-65.

Jones, I. L. & Hunter, F. M. 1993. Mutual sexual selection in a monogamous seabird. *Nature,* **362**: 238-239.

Kempenaers, B., Verheyen, G. R. &. Dhondt, A. A. 1995. Mate guarding and copulation behavior in monogamous and polygynous blue tits. Do male follow a best of a bad job strategy. *Behavioral Ecology and Sociobiology,* **36**: 33-42.

Kempenaers, B., Verheyen, G. R. & Dhondt, A. A. 1997. Extrapair paternity in the blue tit (*Parus caeruleus*): female choice, male characteristics, and offspring quality. *Behavioral Ecology,* **8**: 481-492.

Keyser, A. J. & Hill, G. E. 2000. Structurally based plumage coloration is an honest signal of quality in male blue grosbeaks. *Behavioral Ecology,* **11**: 202-209.

Komdeur, J., Oorebeek, M., van Overveld, T. & Cuthill, I. C. 2005. Mutual ornamentation, age, and reproductive performance in the European starling. *Behavioral Ecology,* **16**: 805-817.

Kokko, H. 1997. Evolutionarily stable strategies of age-dependent sexual advertisement. *Behavioral*

Ecology and Sociobiology, **41**: 99-107.

Kokko, H. 1998. Good genes, old age and life-history trade-offs. *Evolutionary Ecology*, 12: 739-750.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. & Komdeur, J. 2007. The evolution of mutual ornamentation. *Animal Behaviour,* **74**: 657-677.

Kraaijeveld, K., Gregurke,J., Hall,C., Komdeur, J. & Mulder, R. A. 2004. Mutual ornamentation, sexual selection, and social dominance in the black swan. *Behavioral Ecology,* **15**: 380-389.

Lande, R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, **34**: 292-305.

Lande, R. & Arnold, S. J. 1985. Evolution of mating preference and sexual dimorphism. *Journal of Theoretical Biology*, **117:** 651-664.

Laskemoen, T., Fossøy, F., Rudolfsen, G. & Lifjeld, J. T. 2008. Age-related variation in primary sexual characters in a passerine with male age-related fertilization success, the bluethroat *Luscinia svecica. Journal of Avian Biology*, **39**: 322-328.

Lifjeld, J. T. & Robertson, R. J.. 1992. Female control of extra-pair fertilization in tree swallows. *Behavioral Ecology and Sociobiology,* **31**: 89-96.

Lozano, G. A. 1994. Size, condition and territory ownership in male tree swallows (*Tachyneta bicolor*). *Canadian Journal of Zoology,* **72:** 330-333.

Lubjuhn, T. 2005. Consequences of being unfaithful - costs and benefits of extra pair copulations in birds. *Vogelwarte*, **43:** 3-13.

Manning, J. T. 1985. Choosy females and correlates of male age. *Journal of Theoretical Biology*, **116**: 349-354.

Marshall, T. C., Slate, J., Kruuk, L. E. B. & Pemberton, J. M.. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology,* **7:** 639-655.

Møller, A. P. 1988. Female choice selects for male sexual tail ornaments in the monogamous barn swallow. *Nature*, **332:** 640-642.

Møller, A. P., Saino, N., Taramino, G., Galeotti, P. & Ferrario, S. 1998. Paternity and multiple signalling: effects of a secondary sexual character and song on paternity in the barn swallow. *American Naturalist*, **151**: 236-242.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. & Martínez-de la Puente, J. 2007. Female ornaments in the Pied Flycatcher *Ficedula hypoleuca*: associations with age, health and reproductive success. *Ibis*, **149**: 245-254.

Muma, K. E. & Weatherhead, P. J. 1989. Male traits expressed in females: direct or indirect selection?. *Behavioral Ecology and Sociobiology*, **25**: 23-31.

Nicolaus, M., Le Bohec, C., Nolan, P. M., Gauthier-Clerc, M., Le Maho, Y., Komdeur, J. & Jouventin, P.. 2007. Ornamental colours reveal age in the king penguin. *Polar Biology*, **31**:53–61.

Norris, K. 1993. Heritable variation in a plumage indicator of viability in male great tits *Parus major*. *Nature*, **362**: 537-539.

Ortego, J., Aparicio, J. M., Calabuig, G. & Cordero, P. J. 2007. Risk of ectoparasitism and genetic diversity in a wild lesser kestrel population. *Molecular Ecology*, **16**: 3712-3720.

Otter, K., Ratcliffe, L., Michaud, D. & Boag, P. T. 1998. Do female black-capped chickadees prefer high-ranking males as extra-pair partners?. *Behavioral Ecology and Sociobiology*, **43**: 25-36.

Petrie, M. 1994. Improved growth and survival of offspring of peacocks with more elaborate traits. *Nature*, **371**: 598-599.

Pilastro, A., Griggio, M., Biddau, L. & Mingozzi, T. 2002. Extra-pair paternity as a cost of polygyny in the rock sparrow: behavioural and genetic evidence of the trade-off hypothesis. *Animal Behaviour*, **63**: 967-974.

Pomiankowski, A. & Iwasa, Y. 1998. Runaway ornament diversity caused by Fisherian sexual selection. *Proceedings of the National Academy of Sciences of U.S.A*, **95:** 5106-5111.

Richardson, D. S. & Burke, T. 1999. Extra-pair paternity in relation to male age in Bullock's orioles. *Molecular Ecology,* **8**: 2115-2126.

Richardson, D. S., Jury, F. L., Dawson, D. A., Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in *Silviidae* species and their cross-species amplification in other passerine birds. *Molecular Ecology*, **9**: 2226-2231.

Romero-Pujante, M., Hoi, H., Blomqvist, D. & Valera, F. 2002. Tail length and mutual mate choice in bearded tits. *Ethology*, **108**: 885-895.

Rubenstein, D. R. 2005. Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*. *Molecular Ecology*, **5**: 739-744.

Schmoll, T., Mund, V., Dietrich-Bischoff, V., Winkel, W. & Lubjuhn T. 2007. Male age predicts extrapair and total fertilization success in the socially monogamous coal tit. *Behavioral Ecology,* **18**:1073-1081.

Sheldon, B. C., Merila, J., Qvarnstrom, A., Gustafsson, L. & Ellegren, H. 1997. Paternal genetic contribution to offspring condition predicted by size of male secondary sexual character. *Proceedings of the Royal Society of London, Series B,* **264**: 297-302.

Siefferman, L. & Hill, G. E. 2003. Structural and melanin coloration indicate parental effort and reproductive success in male eastern bluebirds. *Behavioral Ecology,* **14**: 855-861.

Smith, H.G. & Vonschantz, T. 1993. Extra-pair paternity in the European starling-the effect of polygyny. *Condor*, **95**: 1006-1015.

Stutchbury, B. J. 1994. Competition for winter territories in a neotropical migrant- the role of age, sex and color. *Auk,* **111**: 63-69.

Sundberg, J. & Larson, C. 1994. Male coloration as an indicator of parental quality in the yellowhammer, *Emberiza citrinella. Animal Behaviour,* **48**: 885-892.

Sundberg, J. & Dixon, A. 1996. Old, colourful male yellowhammers, *Emberiza citrinella*, benefit from extra-pair copulations. *Animal Behaviour,* **52**: 113-122.

Tabachnick, B. G. & Fidell, L. S. 2007. *Using Multivariate Statistics.* 4th edn. Boston: Allyn & Bacon Inc.

Tarof, S. A., Dunn, P. O. & Whittingham, L. A.. 2005. Dual functions of a melanin-based ornament in the common yellowthroat. *Proceedings of the Royal Society of London, Series B,* **272**: 1121-1127.

Trivers, R. L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (Ed. by B. G. Campbell.), 139-179. Chicago: Aldine.

Van Dongen, W. F. D. & Mulder, R. 2007. Relative importance of multiple plumage ornaments as status signals in golden whistlers (*Pachycephala pectoralis*). *Behavioral Ecology and Sociobiology,* **62**: 77-86.

Van Doorn, G. S. & Weissing, F. J. . 2004. The evolution of female preferences for multiple indicators of quality. *American Naturalist,* **164**: 173-186.

Veiga, J. P. 2000. Infanticide by male birds. In: *Infanticide by Males and its Implications.* 1st edn. (Ed. by C. P. van Schaik, and C. H. Janson.), pp 198-220. Cambridge: Cambridge University Press.

Veiga, J. P. & Puerta, M. 1996. Nutritional constraints determine the expression of a sexual trait in the house sparrow, *Passer domesticus. Proceedings of the Royal Society of London, Series B,* **263**: 229-234.

Veiga, J. P., Moreno, J., Cordero, P. J. & Mínguez, E. 2001. Territory size and polygyny in the Spotless Starling: Resource-holding potential or social inertia?. *Canadian Journal of Zoology,* **79**: 1951-1956.

Veiga, J. P., Moreno, J., Arenas, M. & Sánchez, S. 2002. Reproductive consequences for males of paternal vs territorial strategies in the polygynous Spotless Starling under variable ecological and social conditions. *Behaviour,* **139**: 677-693.

Veiga, J. P., Polo, V. & Viñuela. J. 2006. Nest green plants as a male status signal and courtship display in the Spotless Starling. *Ethology,* **112**: 196-204.

Wagner, R. H., Schug, M. D. & Morton, E. S. 1996. Condition-dependent control of paternity by female purple martins: implications for coloniality. *Behavioral Ecology and Sociobiology*, **38**: 379-389.

Weatherhead, P. J. 1984. Mate choice in avian polygyny- Why do females prefer older males. *American Naturalist,* **123**: 873-875.

Weatherhead, P. J. & Boag, P. T. 1995. Pair and extra-pair mating success relative to male quality in red-winged blackbirds. *Behavioral Ecology and Sociobiology*, **37**: 81-91.

Westerdahl, H., Waldenstrom, J., Hansson, B., Hasselquist, D., von Schantz, T. & Bensch, S. 2005. Associations between malaria and MHC genes in a migratory songbird. *Proceedings of the Royal Society of London, Series B,* **272**: 1511–1518.

Westneat, D. F. 1993. Polygyny and extrapair fertilization in Eastern red-winged blackbirds (*Agelaious phoeniceus*). *Behavioral Ecology,* **4**: 49-60.

Westneat, D. F., Sherman, P. W. & Morton, M. L. 1990. The ecology and evolution of extrapair copulations in birds. In: *Current ornithology,* vol 7, (Ed. by D. M. Power), pp 331-369. New York: Plenum Press.

Yezerinac, S. M. & Weatherhead, P. J. 1997. Extra-Pair Mating, Male Plumage Coloration and Sexual Selection in Yellow Warblers (*Dendroica petechia*). *Proceedings of the Royal Society of London, Series B,* **264**: 527-532.

Zahavi, A. 1975. Mate selection: a selection for a handicap. *Journal of Theoretical Biology,* **53**: 205-214.

Zuk, M., Thornhill, R., Ligon, J. D., Johnson, K., Austad, S., Ligon, S. H., Thornhill, N. W. & Costin, C. 1990. The role of male ornaments and courtship behavior in female mate choice of red jungle fowl. *American Naturalist,* **136**: 459-473.

CAPÍTULO 5

Posibles beneficios genéticos de la promiscuidad para las hembras de estornino negro

Elena García-Vigón, José Pablo Veiga & Pedro J. Cordero

RESUMEN

Varias hipótesis no mutuamente excluyentes se han propuesto para explicar la paternidad extra-pareja (EPP) desde el punto de vista de los beneficios genéticos que ésta puede tener para las hembras: hipótesis de diversidad genética, hipótesis de garantía de la fertilidad, hipótesis de los "buenos genes" e hipótesis de la compatibilidad genética. Tener en cuenta las predicciones de estas hipótesis es crucial para distinguir entre ellas y esclarecer cuál podría mantener la estrategia de la EPP en el estornino negro. Nuestros estudios previos en esta especie han mostrado un efecto de los caracteres fisiológicos y morfológicos de las hembras sobre las tasas de EPP. En este estudio analizamos los correlatos de la EPP en relación a todas estas hipótesis intentando averiguar algo más sobre los determinantes del apareamiento promiscuo en aves. Analizamos los patrones de paternidad extra-pareja a lo largo de seis años teniendo en cuenta la similitud genética de los miembros de la pareja e hicimos análisis a diferentes niveles (pareja, macho, pollada y pollo) para testar cada una de las predicciones surgidas de las distintas hipótesis propuestas. Usamos caracteres genéticos, morfológicos y de historia de vida de hembras reproductoras, machos reproductores y pollos. Encontramos que la similitud de las parejas sociales cuyas polladas contenían pollos extra-pareja (EPOs) era más alta que la de las parejas sociales cuyas polladas no contenían EPOs y que el tamaño de puesta era mayor en las polladas de paternidad mixta que en las polladas con un único padre genético. Nuestros resultados sugieren que una de las hipótesis más plausibles para explicar la EPP como una estrategia reproductora alternativa desde el punto de vista de las hembras en esta especie es la hipótesis de la diversidad genética.

Palabras clave: Tamaño de puesta, paternidad extra-pareja, hipótesis de diversidad genética, microsatélites, análisis de paternidad, *Sturnus unicolor*.

Possible genetic benefits of female promiscuity in the spotless starlings

ABSTRACT

Several non-mutually exclusive hypotheses have been proposed to explain the benefits that extra-pair paternity (EPP) may have to females: the genetic diversity hypothesis, the fertility insurance hypothesis, the good genes hypothesis and the genetic compatibility hypothesis. Taking into account the predictions of these hypotheses is crucial to discern among them and envisage which could hold for the EPP strategy in the spotless starling. Our previous studies in this species have shown an effect of female physiological and morphological traits on EPP rates. In the present study we analyse the correlates of EPP in relation to all these hypotheses trying to know something more about the determinats of the promiscuous breeding in birds. We analysed the pattern of EPP along six years having into account pair relatedness and we made analyses at different levels (pair, male, brood and offspring) to test each one of the predictions arisen from the different hypotheses proposed. We used genetic, morphologic and life-history traits of breeding females, breeding males and offspring. We found that relatedness of social pairs whose broods contained extra-pair offspring (EPOs) was higher than relatedness of social pairs whose broods did not contain EPOs and clutch size was higher in mixed paternity broods than in broods with only one genetic father. Our results suggest that one of the most plausible hypotheses to explain the EPP as an alternative reproductive strategy from the female point of view in this species is the genetic diversity hypothesis.

Keywords: Clutch size, extra-pair paternity, “genetic diversity” hypothesis, microsatellites, paternity testing, *Sturnus unicolor*.

INTRODUCTION

Extra-pair paternity (EPP) is a common phenomenon among birds (Petrie & Kempenaers

1998). Among passerines, 86% of the species investigated produce extra-pair offspring (EPO) (Griffith *et al.* 2002). However, some reviews have shown that there is a large variation in the frequency of EPP, both between and within species, and they have highlighted our limited understanding of the possible function of this reproductive strategy to female birds (Griffith *et al.* 2002; Westneat & Stewart 2003; O'Brien & Dawson 2007; Dreiss *et al* 2008). To date, several hypotheses have tried to explain why female birds would solicit or accept extra-pair copulation (EPC) in spite of the costs incurred by this behaviour (Westneat *et al.* 1990; Kempenaers & Dhondt 1993; Petrie & Kempenaers 1998; Jennions & Petrie 2000; Griffith *et al.* 2002). All these hypotheses are based on the direct or indirect benefits that EPP could have for females. For example, females could obtain direct benefits accepting to copulate with extra-pair males in exchange of being allowed to forage inside extra-pair male's territory, obtaining help in nest defence (Gray 1997) or securing food provisioning for the chicks (Blomqvist *et al.* 2005). However, the existence of direct fitness benefits for females is equivocal despite extensive tests for demonstrating such effects (Birkhead & Møller 1992). Females are currently more believed to gain indirect or genetic benefits through EPC by improving their fertility or the average genetic quality of their offspring (reviews in Kempenaers & Dhondt 1993; Jennions & Petrie 2000; Griffith *et al.* 2002; Chamantier *et al.* 2004).

Several non-mutually exclusive hypotheses have been proposed to explain the EPP from the point of view of the genetic benefits to females. The main hypotheses are the genetic diversity hypothesis (Williams 1975; Westneat *et al.* 1990), the fertility insurance hypothesis (Wetton & Parkin 1991; Sheldon 1994), the good genes hypothesis (Hamilton 1990; Westneat *et al* 1990) and the genetic compatibility hypothesis (Zeh & Zeh 1996, 1997). The genetic diversity hypothesis proposes that females engage in EPC for maximizing the genetic variability of their offspring, increasing the probability that some offspring will survive, especially in unpredictable and fluctuating environments, favouring different genotypes under different circumstances. This hypothesis predicts: 1) few EPOs per nest in most nest or, if many EPOs per nest, they should have different fathers, 2) no difference in average fitness between EPOs and WPOs, 3) a higher average fitness in broods with EPOs (mixed broods) than in broods with only one genetic father, 4) most or all females should engage in EPCs, 5) the variance in male reproductive success in the population should not increase, 6) EPP should be more common if pair members are relatives and 7) In contrast with the "good genes" hypothesis and with the "compatible genes" hypothesis, females would not be able to assess genetic quality of extra-pair males. An evidence for an effect of genetic diversity on EPP was found by Petrie *et al.* (1998) who reported a positive correlation between frequency of EPP and genetic variability across species and populations when using the number of polymorphic loci in different species as a measure of genetic diversity.

The "fertility insurance" hypothesis proposes that females could accept EPCs for insuring successful fertilization of their eggs. This could be especially important in species with polygynous mating systems, where males mated to many females simultaneously may suffer from temporary sperm depletion, thus becoming temporarily infertile (Edler & Frield 2008). This hypothesis predicts: 1) higher number of EPO in broods of polygynous males in case of temporal sperm depletion, 2) no average fitness differences between EPOs and WPOs, 3) higher hatching success in broods with mixed paternity than in broods lacking EPOs, and 4) no phenotypic differences between extra-pair males and social males.

The "good genes" hypothesis proposes that females try to pair with males who posses a pool of "good genes" that could be inherited by their offspring improving their survival (Hasselquist *et al.* 1996; Kempenaers *et al.* 1997) and/or their future reproductive performance (Schmoll *et al.* 2005). Predictions related with this hypothesis are: 1) females are able to recognize males carrying these genes using phenotypic cues, for example secondary sexual characters (Hagelin & Ligon 2001, Doucet 2002), 2) higher rates of EPO in the nest of less attractive/fitted males, 3) differences between extra-pair males and the males they cuckolded, being the males who exhibit a higher genetic quality the extra-pair males and 4) differences between EPOs and WPOs from the same brood, with a higher genetic quality of EPOs. Several studies have supported this hypothesis showing differences between cuckolders and the males they cuckolded (e.g. Kempenaers *et al.* 1992; Hasselquist *et al.* 1995; Weatherhead & Boag 1995; Sundberg & Dixon 1996; Kempenaers *et al.* 1997; Saino *et al.* 1997; Sheldon *et al.* 1997; Otter *et al.* 1998, Kokko 2001). Others did not it (Krokene *et al.* 1998, Strohbach *et al.* 1998, Rosivall *et al.* 2009).

Finally, the "genetic compatibility" hypothesis proposes that females could pair with extra-pair males whose genome best complement their own, not only for trying to avoid inbreeding depression but also to maximize the genetic quality of their offspring resulting in a great offspring heterozigosity. There are several examples in birds in which heterozygosity correlates positively with survival, low risk to be parasitized, territory size, song diversity, male plumage characters, clutch size, hatching success fledging success, and fertilization success (Hansson *et al.* 2001; Foerster *et al.* 2003; Cordero *et al.* 2004; Seddon *et al.* 2004; Ortego *et al.* 2007a,b). This hypothesis predicts: 1) higher EPO rates in pairs with higher genetic relatedness, 2) higher relatedness between female and her social mate than between the female and her extra-pair male and 3) higher levels of inbreeding and lower levels of heterozigosity in WPOs than in EPOs from the same brood. Some relative recent studies have supported this hypothesis (e.g. Blomqvist *et al.* 2002; Foerster *et al.* 2003; Freeman-Gallant *et al.* 2006; Fossoy *et al.* 2008).

As we show, many studies have tested the different hypotheses about the function of EPP from the point of view of females at the intra-specific level obtaining contradictory results. As Griffith *et al.* (2002) pointed out, good empirical tests require a large body of information on individual fitness and quality of females, males and offspring. We used EPP data from a colony of spotless starlings (*Sturnus unicolor*) collected along six years in an attempt to provide new insights concerning this behaviour. In previous studies, we have already shown that EPP is a common strategy in this species (Cordero *et al.* 2003) and that female physiological and morphological traits influence the rate of EPO in the colony (García-Vigón *et al.* 2008b; García-Vigón *et al.* unpublished).

However, in none of these studies, we analysed together and in detail which of the several hypotheses potentially underlying extra-pair choice could better fit our model species. So, to attain this, in the present study we analyse the correlates of EPP in the spotless starling testing the predictions of the different genetic hypotheses trying to get additional insights into the intriguing breeding strategy of avian promiscuity. For this purpose, we used genetic, morphologic and life-history traits of breeding females, breeding males and offspring. We analysed the pattern of EPP along six years having into account pair relatedness and we compared social pairs with and without EPOs in their broods, social pairs versus extra-pair mating pairs, cuckolder males versus males they cuckolded and broods with and without mixed paternity. Finally, we also compared EPOs versus WPOs from the same broods, and therefore individuals exposed to the same environmental conditions and originated from the same maternal genotype (Sheldon *et al.* 1997).

METHODS

Species and Study Area

The spotless starling is a mid-size facultative polygynous passerine in which, depending on the year, 25%-45% of males that acquire a breeding territory mate with two or more females in our population and the remaining males acquire a single mate (Veiga *et al.* 2001 2002). The laying season extends from mid April to mid-June and most females lay two clutches per breeding season (Mean = 4.9 eggs per clutch). Both males and females defend nest against competitors and intrasexual competition for nest sites is intense in both sexes (Veiga 2002). Previous studies have shown that in this species between 10-20% of the offspring is sired by extra-pair males (Cordero *et *al.* 2003; García-Vigón *et al.* 2008b).

We studied spotless starlings in a colony at Collado Villalba (Madrid, Spain) consisting of 40 nestboxes in a plot of 1.3 ha. The area is pastureland with trees. The study of this colony has been underway since 1995 (Veiga *et al.* 2004) and data for this study was gathered along 6 years (1996-2001).

Field procedures

Nestboxes were checked to determine the beginning of laying, clutch size, hatching date and number of fledglings. A proportion of the breeders had already been marked in 1995 and they were individually recognized. The unmarked males were in most cases captured before egg laying using spring nest traps but some of them could only be trapped when they fed young. To identify the male that controlled a nestbox we observed nestboxes during prelaying stages or, if a male fed nestlings, we trapped him or identified his rings on nest video recordings. The identity of female owners of nest boxes was determined in most cases by capturing them when they had nestlings 4-6 days old (Moreno *et al.* 1999; Veiga *et al.* 2002). In 1996 and 1997, we validated this criterion by verifying that the presumed owners were the genetic fathers of at least some offspring in their nest using DNA multilocus fingerprinting (Cordero *et al.* 2003). In total, we followed 217 successful breeding attempts along six years (1996-2001) from 78 females and 46 males, of which 82.03 % (178/217) produced at least one fledgling.

Standard morphometric measurements (wing length, outermost wing primary length, tarsus length, body mass, bill length and bill height) and a blood sample were taken from captured individuals (García-Vigón *et al.* 2009). Body condition was also computed as typified residuals from linear regressions of body mass on tarsus length. The capture of males and females did not affect either their mating status or their current nestbox occupation. We ringed all adults that were not yet marked with a numbered aluminium ring and a unique combination of plastic colour rings to allow individual identification. Nestlings were ringed with aluminium rings when they were 5-6 days old and then they were bled. All blood samples (50-100µl) were taken from the jugular vein of all captured adults and nestlings, kept in absolute ethanol and stored at -40°C until they were used for parentage and sex analysis.

The sex of young was identified by PCR amplifications of two homologous genes (CHD1-W and CHD1-Z) using primers P2 and P8 and following Griffiths *et al.* (1998) protocol with certain modifications (see Cordero *et al.* 2001).

Measuring Parental Care

We estimated the parental care as the sum of male feeding trips/h (male feeding rate; Allen& Islam 2004) plus female feeding trips/h (female feeding rate). We used parental feeding rate of nestlings instead of other measures of parental care because it is relatively easy to record. Feeding rate of males and females at each nest was measured in 1998, 1999 and 2000 by means of two video camera sessions, each lasting 2 h, when nestlings were 4–5 and 10–12 days old respectively. The average rates for these two periods were used in our analyses (see Moreno *et al.* 1999 for more details of the rationale). Cameras were placed 3–6 m from of the entrance of the nestboxes and observations were made at 08.00–10.00 hours and 16.00–18.00 hours allowing parents identification by their individual combination of colour rings.

Genetic Parentage Assignment, Individual Homozigosity and Mating Pair Relatedness

For years 1996 and 1997, we used parentage data that we had obtained using DNA multilocus fingerprinting in a previous study (Cordero *et al.* 2003). To establish the true genetic relationships among the individuals for the rest of years included in the present study (1998-2001), we carried out microsatellite genotyping on families whose social parentage had been determined previously.

To extract and purify genomic DNA from the blood samples we used NucleoSpin Tissue Kit (Macherey-Nagel, Duren, Germany). We determined the genetic parentage of 54 social males, 81 breeding females and 777 nestlings from 217 broods using the following primers: Pca-7 from blue tit, *Cyanistes* *caeruleus* (Dawson *et al.* 2000), Ase-18 from Seychelles warbler, *Acrocephalus sechellensis* (Richardson *et al.* 2000), and SS2.32, SS1.6, SS1.106, SS2.106 from superb starling, *Lamprotornis superbus* (Rubenstein 2005). These microsatellites were sized using a ROX labelled size standard in a single-capillary automated DNA sequencer (ABI 310 ® Genetic Analyzer, Applied Biosystems) and genotypes were scored using GeneScan 3.7 (Applied Biosystems). Microsatellites were polymorphic for spotless starling with an average of eight alleles per locus (range: 4 - 12) and had a combined exclusion probability of 0.973 (García-Vigón *et al* 2008a). Their suitability for paternity analyses has been already validated in previous studies (e.g. García-Vigón *et al.* 2008 a,b).

We assessed genetic parentage by comparing nestling genotypes firstly to those of the attending female and then to the territorial male at six loci. We considered that nestlings were within-pair offspring (WPO) if their alleles matched (i.e. if they possessed alleles of the size of each parent and segregation was compatible with Mendelian inheritance) the social parents alleles in all six loci or had at the most one mismatched allele, assuming that this mismatch could be originated through mutation or typing error. We considered that nestlings were EPO if their alleles mismatched with those of the social male at two or more loci (García-Vigón *et al.* 2008a, b, c). We consider that nestlings were intraspecific-brood parasites (IBP) when their alleles mismatches with both their social male and social female after discarding samples assignment errors (García-Vigón *et al.* 2008a, b, c). We consider that nestlings were pseudoparasite offspring if their alleles mismatches with those of their social female but not with those of their social male. Only the cases with two or more mismatches, involving the offspring and their putative parents, were genotyped again to confirm the obtained results (García-Vigón *et al.* 2008 a, b). When we removed the locus with more typing errors, the combined exclusion probability still was very high (0.941).

We determined male extra-pair gains of paternity in alien nests in 1998, 1999, 2000 and 2001 by using CERVUS, version 2.0 (Marshall *et al.* 1998) a likelihood-based computer program to assign the most probable genetic paternity of an EPO nestling among a set of different putative males with a level of confidence set at 95%. We run the program with one simulation for each study year. Simulation parameters were: 95.4% of loci typed and 33 candidate parents, of which 82% were sampled for 1998; 99.2% of loci typed and 44 candidate parents, of which 86% were sampled for 1999; 98.9 % of loci typed and 39 candidate parents, of which 87% were sampled for 2000, and 99.4% of loci typed and 34 candidate parents, of which 71% were sampled for 2001. We estimated the number of candidate parents increasing the field data (number of territorial and observed floating males in the colony) by approximately 20% to account for parentage of not sampled individuals (Berg 2005). We run all simulations for 10000 cycles assuming an error rate of 1%, the default value in CERVUS.

We used the program CERNICALIN (Aparicio *et al.* 2006) to calculate three different index of multilocus heterozygosity for each genotyped individual: Observed Homozigosity (H0), Internal Relatedness (IR) (Amos *et al.* 2001) and Homozigosity by Loci (HL) (Aparicio *et al.* 2006). However, all of these three indexes provided similar results, so here only results of HL index are included.

Finally, we needed to estimate relatedness between social and genetic mating pairs and between females and the males present in the colony in a determinate breeding season. Relatedness or genetic similarity between mates is an important and easily measured component of genetic compatibility (e.g. Blomqvist *et al.* 2002; Pai & Yan 2002; Marshall *et al.* 2003) because dissimilarity between parental haplotypes increases the chances of heterozygosity at offspring loci which could be positive for their fitness. Relatedness could influence reproductive patterns through a variety of adaptive and non-adaptive mechanisms. For example, females may choose mates based on relatedness or a correlate of relatedness (e.g. Foerster *et al.* 2003; Marshall *et al.* 2003). Alternatively, postcopulatory mechanisms such as sperm competition, cryptic female choice, or interactions between sperm and ova may result in a higher probability of fertilization of eggs by genetically dissimilar sperm (e.g. Tregenza & Wedell 2002; Pai & Yan 2002; Marshall *et al.* 2003) or differential survival of embryos as a function of the genetic dissimilarity of their parents (Tregenza & Wedell 2002). These different mechanisms can be difficult to tease apart and few studies have successfully identified them (but see Marshall *et al.* 2003). Regardless of the mechanism, reproductive patterns in relation to genetic similarity can indicate the indirect adaptive benefits of multiple mating to females. If selection has favoured mechanisms that enhance the probability that offspring result from genetically dissimilar ova and sperm, the predictions established by the genetic compatibility hypothesis should hold (Griffith *et al.* 2002).

To estimate relatedness we used the program STORM, version 1.0 (Frasier 2008), a program written in C designed to use Monte Carlo simulations in order to test a variety of hypotheses regarding relatedness and/or mating patterns, for calculating relatedness of social and genetic mating pairs. In this program the calculation of relatedness of mating pairs is based on the method described in Li *et al.* (1993), with each locus weighted using the method described by Lynch & Ritland (1999) and Van de Casteele *et al.* (2001). STORM calculates different weights for each pairwise comparison that are based only on the loci used for that comparison, rather than all loci in the data set (Frasier 2008). This method was chosen out of the many available approaches for calculating relatedness because it is unbiased, it is never undefined, and it consistently performs well in a variety of situations and often outperforms all other estimators (Van de Casteele *et al.* 2001; Wang 2002; Krützen *et al.* 2003).

Statistical analyses

We used different statistical units for the analyses (male, pair, brood and offspring) according to the different predictions exposed. At male level, we used paired t-test for making comparisons between cuckolder male(s) and the social male they cuckolded. We compared their relatedness with the social female, their individual homozigosity, their reproductive experience (estimated as the number of years that a male had bred in the colony until the study year), some variables of mating success (male status, number of breeding attempts, number of nest and number of mates) and some phenotypic traits (outermost primary length, wing length, ornament length, tarsus length, body mass and body condition). These traits have been used by many other authors as indicators of male quality (for example Kempenaers *et al.* 1997, Krokene *et al.* 1998, Strohbach *et al.* 1998, Leech *et al.* 2001). For these comparisons, we combined data over all years in which we have unambiguously identified cuckolder males (1998-2001). To avoid pseudoreplication in individual male analyses, if a social male was cuckolded in several breeding attempts in which he was paired with the same social female, we considered this male only one time in the analyses and we compared his average data with the average data of his cuckolder males. There were 23 cases in which a social male had more than one EPO in his brood. We could assign the true genetic father of all EPOs in four of these cases. In one of them the true genetic father was the same for all EPOs. In the other three cases, EPOs in the same brood had different true genetic fathers but, unfortunately, we only had morphological data for one of the identified genetic fathers. So, in such cases, we compared the social male with the genetic father for which we had morphological data. On the other hand, some males sired EPOs in different broods, but each case can be considered as an independent event of female choice (Kempenaers *et al.* 1997, Frield & Klump 2005).

At pair level, we used one way ANOVA to test if there were significant differences in the average relatedness of each breeding female with all males present in the colony in this breeding season, but her social mate, comparing females from social breeding pairs with EPOs and without EPOs respectively. We used the same test to compare the relatedness of social mating pairs whose broods contained EPOs and those that did not. In both cases, we had data for several broods of each mating pair so, to avoid pseudoreplication, we considered only first clutches and we also used a bootstrapping procedure. We run an identical ANOVA model 50 times, each time randomly selecting with replacement one value for each breeding pair and calculating average *F* statistics across the replicates (Yezerinac *et al.* 1996). To check if the obtained *F*-statistics was or not significant we used the probability calculator of the statistical package STATISTICA 6.0 (Statsoft Inc., Tulsa, OK, U.S.A). In addition, and considering only first broods with EPOs, we used Pearson correlations to test if the relatedness of females with their social and extra-pair mates were correlated with the percentage of EPOs in their broods. In this case, pseudoreplication was not a relevant problem because we only had one social pair that had three broods with EPOs and one extra-pair mate that sired EPOs two times. Nevertheless, we followed the same method than above but in this case we only repeated the analyses 3 times for the relatedness between females and their social males and 2 times for the relatedness between females and their extra-pair males (selecting each time one value for the pair). We calculated the average correlation coefficient of Pearson (*r*) across the replicates. To check if the obtained correlation coefficients were or not significant we used again the probability calculator of the statistical package STATISTICA 6.0 (Statsoft Inc., Tulsa, OK, U.S.A). At this level, we also used paired t test for comparing relatedness between a female and her mate (social or extra-pair depending on the analysis) to the average relatedness of that same female and all males present in the population in that year . With this analysis, we try to test if the female is selecting her mates (social or extra-pair) by their genetic similarity from the pool of males in the population (Tarvin *et al.* 2005).

Finally, we investigated the potential benefits that EPP could exert on offspring at both brood and nestling levels. At brood level, we first performed an initial exploratory analysis in which we compared the performance of broods with mixed and non-mixed paternity using one-way ANOVAs in relation to clutch size, hatching success, nestling success (defined as proportion of fledglings relative to clutch size), sex ratio (defined as number of male fledglings in the brood/total number of fledglings in the brood), mean offspring body mass, mean offspring tarsus length and mean offspring body condition index (measured when they were 15 days old) as response variables. Furthermore, to control for variables that were known or suspected to affect these response variables we made a second analyses using ANCOVA models (general linear models, SPSS Inc, Chicago, IL, U.S.A.) in which we included the presence of EPOs in the brood as fixed factor and the same response variables as before, except hatching success and nestling success that were interchanged for number of nestlings and number of fledglings respectively. We controlled for the environmental conditions and the possible effects of testosterone in brood success including year (1996, 1997, 1998, 1999, 2000 or 2001) and female treatment (testosterone or control) as fixed factors provided that we used this hormone in previous work for different studies on this species (Veiga *et al.* 2004; García-Vigón *et al.* 2008). We also included the variables female heterozigosity (Hl index) and pair relatedness as covariates because their potential effects on breeding success (Cordero *et al.* 2004). Depending on the analysed variable, we also included other covariates in the model that could be influential. For example, for clutch size we added female condition as covariate; for number of nestlings we added female condition, clutch size and male status (monogamous or polygynous); for number of fledglings we added number of hatched nestlings and parental feeding rate; and finally, for fledglings measures (body mass, tarsus and body condition) we added parental feeding rate and number of fledglings. Except for the analyses of clutch size, in which the interaction between year and the presence of EPOs and the interaction between treatment and presence of EPOs resulted significant, we selected a main effects model after having verified that all interactions were not significant (all $P > 0.100$). For the analyses of offspring measures, we used only broods with at least one fledgling. Only those broods in which parental feeding was measured were used to analize the number of fledglings and their morphometric variables in the ANCOVA models. We had data from the same pair for several years and for several breeding attempts within the same year so, to avoid pseudoreplication in both types of analyses, we considered only first clutches and we used again the bootstrapping procedure described before for one way ANOVAs.

At nestling level, we investigated if there were differences in the survival to fledgling or in the sex between EPOs and WPOs using binary logistic regression models. First, we included survival to fledgling as the response variable and paternity (EPO or WPO), sex, brood identity, and their interaction as covariates. Then, we repeated the analysis including sex (Male=1 and Female=0) as response variable and paternity, brood identity and their interactions as covariates. The covariates used were not correlated among them (all $P>0.05$). Furthermore, we used paired t-test to compare mean values of other phenotypic traits (wing length, tarsus length, body mass, body condition) and homozigosity index of WPOs and EPOs in each brood containing mixed paternity (73 broods). Such comparisons within broods control for territory quality and social parent quality (Kempenaers *et al.* 1997). It is important to note that some of the morphological traits of the nestlings analysed are more dependent on sex than on paternity. For example, in the spotless starling, we found that, within brood, male nestlings presented higher body sizes than female nestlings. (Male tarsus length= 30.34±0.12mm, female tarsus length= 29.79±0.13mm, t= 3.905, N=36, P<0.001; male body mass=83.12±1.44g, female body mass=80.30±1.14g, t=2.831, N=36, P=0.008). Therefore, we repeated paired t test within broods comparing EPOs with WPOs according to sex. Finally, we made this same analysis but excluding the smaller nestling of each brood to avoid a possible effect of hatching asynchrony in the last nestling size. When there was more than one EPO in the brood, we calculated the average values of their traits for comparing them with those of their maternal half-siblings.

RESULTS

Patterns of paternity and EPP

We studied patterns of paternity along 6 years for a total of 217 broods and 776 offspring. Year by year, we found pseudoparasitism in 0-9.09% of the broods, parasitism in 0-6.06% of the broods and EPP in 24.24-49.09% of the broods. Depending on the year, between 33.33% and 70.59% of males lose paternity and between 8.20% and 19.40% of offspring were sired by an extra-pair male (see Table 1). Most of the broods containing EPOs had only one EPO (68.50%) and the rest (31.50%) had two or more. The probability that a brood contained at least one EPO was unrelated to laying date (one-way ANOVA, $F_{1,216}$= 1.429, P= 0.233) for all broods.

Among EPOs identified using microsatellites (1998-2001), we assigned the genetic father in 50 % (21/42) of cases (See Table 1). It is likely that the other extra-pair males were breeding in the study area (in natural cavities) or just outside the study area for which we did not have blood samples. Sixty two percent (13/21) of the identified males were territorial males breeding in the colony and in 38% (8/21) of cases, the genetic father was a floating male. When we considered only territorial males (social paired males) gaining paternity in other nests, we observed that 38.46% (5/13) were in turn cuckolded by other males. In four broods containing more than one EPO (two EPOs in each one) we were able to identify the genetic father of all EPOs. Only in one of this broods the genetic father was the same male for all EPOs. Most of the males that lost paternity did not gain paternity in alien nests (4 of 5 males (80%) in 1998, 9 of 9 males (100%) in 1999, 7 of 8 males (87.50%) in 2000 and 4 of 7 males (57.14%) in 2001. Males never "exchanged" paternity i.e., fathered offspring in each other's nest. For checking if the fact of gaining paternity was affected for the fact of losing it, we made Yates corrected chi square tests including male gains paternity (yes or no) and male loses paternity (yes or no) as variables for each study year. The tests were not significant (all P>0.050).

There was no association between frequency of EPOs and male status: 28.2% (20/71) of the broods of monogamous males versus 36.4% (50/137) of the broods of polygynous males contained at least one EPO (χ^2_1= 1.45, P = 0.23). We were not sure about the male status in nine broods.

Table 1. Summary of paternity data from a *Sturnus unicolor* colony along 6 six years (1996-2001).

	1996[a]	1997[a]	1998	1999	2000	2001	Total
Total broods analysed	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Total number of nestling analysed	169	201	79	106	122	103	776
Total number of different males analysed	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Percentage (number) of broods with IBP	0.00(0)	0.00 (0)	0.00 (0)	6.06 (2)	3.03 (1)	0.00 (0)	1.38 (3)
Percentage (number) of broods with pseudoparasite offspring	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Percentage (number) of broods with EPO	25.53(12)	49.09 (27)	28.57 (6)	33.33 (11)	24.24 (8)	32.14 (9)	33.64 (73)
Percentage (number) of males that lost paternity	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Percentage (number) of EPO	8.28 (14)	19.40 (39)	8.86 (7)	13.21 (14)	8.20 (10)	10.68 (11)	12.24 (95)
Percentage (number) of EPO per brood (Mean±SD)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Percentage (number) of broods with EPO with more than one EPO	16.67 (2)	44.44 (12)	16.67 (1)	27.27 (3)	37.50 (3)	22.22 (2)	31.51 (23)

	1996[a]	1997[a]	1998	1999	2000	2001	Total
Percentage (number) of broods with EPO where biological fathers [illegible] successfully determined	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Percentage (number) of EPO that could be assigned to a male	_	_	14.29 (1)	35.71 (5)	60.00 (6)	81.82 (9)	50 (21)

[a] Genetic paternity assessed through DNA multilocus fingerprinting

Cuckolder males versus males they cuckolded

Among promiscuous pairings, we found no significant differences between cuckolders and males they culckolded neither in genetic relatedness with the female nor between individual homocigosity, morphometric measurements, breeding success (number of females or nest obtained this breeding season) or reproductive experience (Table 2).

Table 2. Pairwise comparisons of extra-pair and within-pair male traits siring young in the same brood.

Character	Within-pair males[a]	Extra-pair males[a]	*N*	*t*	*P*
Relatedness with female	0.06±0.08	-0.01±0.09	15	0.500	0.625
Homozigosity index Hl	0.35±0.05	0.28±0.04	15	1.064	0.306
Wing length	135.86±1.25	135.36±0.97	7	0.491	0.641
Outermost primary feather length	101.00±1.03	100.07±1.01	7	0.932	0.387
Tarsus length	30.49±0.22	30.11±0.19	7	1.069	0.326
Body mass	91.86±1.69	90.21±2.49	7	0.702	0.509
Body condition[b]	-0.08±0.36	0±0.35	7	-0.205	0.845
Ornament length	35.66±1.34	33.97±2.09	6	1.032	0.349
Status	1.40±0.13	1.13±0.17	15	1.468	0.164
Number of mates	2.00±0.17	1.53±0.24	15	1.522	0.150
Number of nests	1.53±0.17	1.13±0.17	15	1.871	0.082
Number of breeding attemps	2.60±0.21	2.20±0.34	15	1.065	0.305
Reproductive experience	2.73±0.43	2.53±0.40	15	0.509	0.619

[a] Means±SE.

[b] Residuals

Pair relatedness and EPO

Relatedness was higher among social mating pairs whose broods contained EPOs (0.05±0.06) than among those social pairs without EPOs (-0.08±0.03) ($F_{1,68}$=5.335, *P*= 0.024; Fig. 1). However, we did not find an association between social pair relatedness and the percentage of EPOs in the broods when only the broods with EPOs were considered (*Pearson correlation coefficient*= 0.069, *P*= 0.717, *N*=30). Relatedness of females and their extra-pair mates neither varied with the percentage of EPOs in the brood (*Pearson correlation coefficient*= -0.300, P= 0.401, N=10).

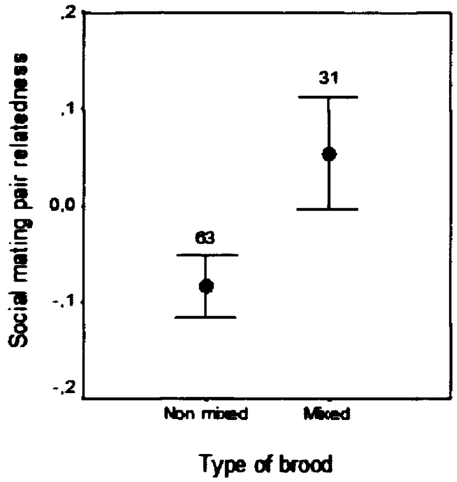

Figure 1. Social pair relatedness (Mean± 1SE) in broods with mixed and non-mixed paternity. The number of broods is given above the points

On the other hand, we did not find differences in the average relatedness of female with all males genotyped in the population (except their social mate) when comparing females whose broods contained EPOs and those that did not ($F_{1,50}$=0.679, P=0.414). Also, relatedness of female with her social mate did not differ from her average relatedness with all other males in the population (paired t tests: t_{68}= -1.095, P= 0.277) even when we considered only females with EPOs in their broods. The same was true when we compared female relatedness to her extra-pair mate versus their average relatedness to other males in the population (paired t test: t_{14}=0.657, P=0.522) suggesting that females are not particularly an extra-pair male according to their genetic similarity among all the males present in the population.

Mixed versus Non-mixed paternity broods

We did not find differences in the proportion of broods containing unhatched eggs when we compared broods with mixed paternity (18/46) and broods without EPOs (34/82; $\chi^2{}_1$ = 0.07, P=0.797, N=128 broods corresponding to first clutches). Using one-way ANOVAs, we neither found significant differences in performance between broods with mixed paternity and those lacking EPOs, although almost all values were slightly higher for broods with mixed paternity. (Table 3).

Table 3. Characteristics of mixed and non-mixed broods

Character	Non-Mixed Broods [a]	Mixed Broods [a]	*F*	g.l.	*P*
Clutch size	4.73± 0.09	5.04± 0.14	2.816	1,102	0.096
Hatching success (percentage of hatched eggs relative to clutch size)	0.88± 0.02	0.91± 0.02	0.453	1,102	0.502
Nestling success (percentage of fledglings relative to clutch size)[c]	0.58± 0.03	0.64± 0.04	0.809	1,102	0.371
Sex ratio	0.47± 0.05	0.48± 0.05	0.139	1,45	0.711
Mean offspring body mass [c]	84.18± 0.65	82.26± 1.51	2.628	1,93	0.108
Mean offspring tarsus length [c]	30.27± 0.06	30.93± 0.74	1.340	1,93	0.250
Mean offspring body condition [b c]	0.38± 0.10	0.41± 0.14	0.241	1,47	0.625

[a] Mean± SE

[b] Residuals

[c] Analysed in broods with fledglings only

However, when we controlled for some influential variables (year, female treatment, female condition, female heterozigosity and pair relatedness), we obtained that clutch size of broods with mixed paternity was significantly higher than clutch size of broods with non-mixed paternity ($F_{1,67}$=11.678, P=0.001, Fig 2).

For the rest of analysed variables after controlling for the possible influential variables, there were not significant differences between broods with and without EPO (Tables are shown in Appendix 1).

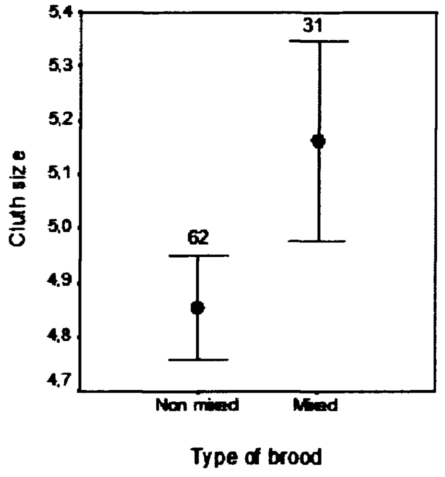

Figure 2. Clutch size (Mean± 1SE) in broods with mixed and non-mixed paternity. The number of sampled broods is given above the points.

EPO versus WPO

Considering 41 broods with mixed paternity where part of the nestlings died before becoming fledglings and using binary logistic regression models, we showed that paternity was not a significant variable for the survival of nestlings. In other words, the proportion of fledgling survival was similar between EPOs and WPOs (57.69% (30/52) of EPOs versus 56.30% (67/119) of WPOs survived) (*B*= -2.057, *Wald*=1.171, *P*=0.279). Furthermore, using the same statistical method, we found that in 63 broods with mixed paternity, the sex of nestlings was not affected for being EPO or WPO (48.61% (35/72) of EPOs versus 48.72% (76/156) of WPOs were males) (*B*= 0.937, *Wald*=2.163, *P*=0.141). In fact, none of the variables used in the models resulted significant for nestling survival or their sex (all *P*>0.05).

We neither found any significant difference in the morphological traits measured nor in the homozigosity index between EPOs and WPOs (Table 4). The absence of significant differences between EPOs and WPOs from the same brood was consistent even when we compared nestlings of the same sex (Table 5) and when we further removed the smaller nestlings of the broods (Table 6).

Table 4. Pairwise comparisons of EPO and WPO traits from the same brood.

Character	WPO[a]	EPO	*N*	*t*	*P*
Wing length	53.39±4.98	53.32±5.64	44	0.093	0.926
Tarsus length	30.01±0.89	30.02±0.95	44	-0.077	0.939
Body mass	81.26±6.72	80.68±9.89	44	0.577	0.567
Body condition[b]	-0.06±0.92	-0.09±0.98	44	0.287	0.775
Hl	0.30±0.13	0.35±0.19	33	-0.994	0.327

[a] Means±SE

[b] Residuals

Table 5. Pairwise comparisons of EPO and WPO traits for the same sex and brood.

Character	WPO[a]	EPO[a]	*N*	*t*	*P*
Wing length	53.42±6.34	53.59±7.54	33	0.194	0.847
Tarsus length	30.80±5.47	30.99±5.31	33	0.982	0.334
Body mass	79.82±12.10	78.68±14.25	32	-0.972	0.338
Body condition[b]	-0.03±1.03	-0.13±1.39	32	-0.658	0.516
HI	0.31±0.19	0.38±0.18	29	1.260	0.218

[a]Means±SE
[b]Residuals

Table 6. Pairwise comparisons of EPO and WPO traits for the same sex and brood removing the smaller nestling.

Character	WPO[a]	EPO[a]	*N*	*t*	*P*
Wing length	54.63±6.17	54.17±7.56	26	-0.454	0.654
Tarsus length	31.13±6.14	31.36±5.94	26	1.064	0.297
Body mass	79.33±17.28	78.16±13.61	25	-1.210	0.238
Body condition[b]	-0.13±1.03	-0.07±0.98	25	-0.549	0.588
HI	0.36±0.17	0.38±0.17	25	0.434	0.669

[a]Means±SE
[b]Residuals

DISCUSSION

We found that relatedness was higher in social pairs whose broods contained EPOs than in those which did not contain EPOs. We also found that clutch size was higher in mixed paternity broods than in broods with only one genetic father. The results on patterns of EPP as the percentage of broods with EPO, proportion of EPO in the broods, characteristics of social and extra-pair males, etc., obtained along six years of study in a colony of spotless starlings, suggest that one of the most plausible hypotheses to explain EPP as an alternative reproductive strategy from the female point of view in this species is the genetic diversity hypothesis. Thus, according with its prediction of few EPO per nest in most nest or, if many EPOs per nest, they should have different fathers, we found that the percentage of broods containing EPOs was never lower than 24% in none of the analysed breeding seasons and most of the broods containing EPO contained only one EPO. In the case that there were more than one EPO per brood, they used to be from different genetic father. Furthermore, the identification of the genetic father revealed that the same male could lose paternity in his own nest and gain it outside it in the same breeding season, so the reproductive success of extra-pair males was not necessarily higher than the reproductive success of the social males they cuckolded, as the genetic diversity hypothesis predicts. On the other hand, the identification of the genetic father also showed that extra-pair males could be floaters or territorial, polygynous or monogamous, previous or new mates for the female, and so on. In other words, we could not identify specific traits of extra-pair males. In fact, in a previous study (García-Vigón *et al.* unpublished, Chapter 4) we found that males gaining paternity in alien nest were similar in age and morphology to non-promiscuous males and, in the present study, we did not find any significant differences between genetic and social fathers.

All these results taken together suggest that female spotless starlings do not seek particular phenotypic traits for choosing a high quality male as extra-pair mate. That is, more than seeking a standard male that can enhance the quality of their offspring through the inheritance of his atributes, as the "good genes" hypothesis proposes, females could seek or accept EPCs from very different males trying to increase the genetic variability or the heterozigosity of their offspring and thus, their viability (Brown 1997). Evidences of this pattern have been already found in several species (Foerster *et al.* 2003; Masters *et al.* 2003; Bishop *et al.* 2007; Suter *et al.* 2007). Females can increase the genetic variability of their offspring by three ways: mating to males that are highly heterozygous, mating to more than one male or mating to males that are genetically different from themselves (Masters *et al.* 2003). In the spotless starling we did not find significant differences in the homozigosity index between social and extra-pair males and therefore the first and the last options are unlikely in this species. However, we know that a lot of spotless starling females are mated with more than one male and that social pairs that have EPOs in their broods have higher relatedness than social pairs that did not have EPOs. This suggests that spotless starling females try to increase the genetic variability of their offspring by pairing with extra-pair males, especially when the genetic relatedness of the social pair is high. Therefore, not only the genetic diversity hypothesis but also the compatible genes hypothesis could explain the promiscuous strategy of this species. However, although pair relatedness could be important to decide to acquire an extra-pair mate, females do not seems to use this character to choose. Thus, we did not detect significant differences in relatedness with the female between her social and her extra-pair mates, which is one of the main predictions of the compatible genes hypothesis. Furthermore other predictions of this hypothesis were neither fulfilled in this study. For example, we did not find differences in mates relatedness when we compared social pairs versus extra-pair mates and neither when we compared social pair relatedness versus average relatedness of a female with all the males present in the colony. So, the genetic diversity hypothesis seems to be more relevant than the compatible genetic hypothesis for explaining EPP from the female point of view in this colony of spotless starlings.

The genetic diversity hypothesis also predicts a better success of broods with mixed paternity than of broods without mixed paternity. According to this, we found that clutch size was larger in broods with than without EPOs. However, a larger clutch size did not reflect a higher breeding success, as we could not detect any other difference between broods with and without mixed paternity in the traits examined (fledgling success, sex ratio, average morphometric measures or average heterozigosity index of the offspring). Anyway, we cannot discard here an effect of the small sample size used in the paired t test. It is known that genetic fitness benefits of mate choice are generally small (Møller & Alatalo 1999) and, therefore, even studies with reasonable sample sizes may fail to detect genetic effects due to a lack of statistical power (Griffith *et al.* 2002; Schmoll *et al.* 2003). Another explanation for the absence of these expected differences may be that genetic benefits of extra-pair mate choice by females are context dependent (see Schmoll *et al.* 2003, 2005). Thus, in environments with favourable conditions, the expected difference in the performance between broods with and without EPOs may be masked and, hence, remain undetected, because a beneficial environment is likely to decrease the significance of heritable paternal genetic variation for offspring fitness. Finally, we must not forget that the absence of differences in the homozigosity indexes between broods with and without EPOs may be merely reflecting that higher fitness benefits are obtained with intermediate values of heterozigosity in the spotless starling (Aparicio *et al.* 2001). We can neither discard here an effect of the low number of loci used for the genetic analyses. Furthermore, in a previous study (García-Vigón *et al.* 2009), we found that social males that lose paternity fed more than males that did not lose paternity. This suggests a higher value of the broods with more genetic variability among nestlings, although there is no a current clue suggesting that males could recognize their genetic offspring.

In relation to the offspring, we neither found significant differences when we compared survival, sex or other morphologic traits and homozigosity indexes between EPOs and WPOs from the same brood. These results agree with the predicions of the genetic variability hypothesis as a possible explanation for the maintaining of EPP, since it states that there is not an optimum genotype, but it depends on environmental conditions. Furthermore, these results were consistent even when we compared individuals from the same sex and when we remove the smaller nestling of the brood that could introduce bias in our analyses.

In sum, all these results suggests that one of the most plausible explanations for the existence of EPP in the spotless starling is given by the genetic variability hypothesis, since most of its predictions were fulfilled in our study. For the remaining hypotheses, we conclude that 1) the absence of a standard male prototype suitable for all the females is not compatible with the "good genes" hypothesis, 2) the fertility insurance hypothesis is not likely for the spotless starling if we have into account that we did not find any association between male status and the percentage of EPOs in their broods, and neither a higher hatching success in broods with mixed paternity.

On the other side, direct or non-genetic benefits derived from extra-pair fertilizations seems unlikely for female spotless starlings given the behaviour of this species. First, using the extra-pair male territory is unlikely for female spotless starlings since in this species nest box owners share open feeding areas, and even neighbors feed in flocks. Nest defence by the extra-pair male seems also unlikely because only social pairs have been observed participating in this activity (J.P. Veiga personal observation). Finally, extra-pair males have never been observed helping non-social mates in feeding nestlings in the spotless starling. Furthermore, in a previous study of this species, Moreno *et al.* (1999) showed that males that fed less rised the same number of nestlings than those feeding at higher rates. This suggests that male help in feeding nestlings does not represent a critical benefit for females.

ACKNOWLEDGEMENTS

We thank Marta Arenas, Sara Sánchez and Eduardo Mínguez for their help during the field work. We acknowledge José Miguel Aparicio for his inestimable help with Excel macros for the generation of samples to avoid pseudoreplication and for the program Cernicalín. We are also very gratefull to Joaquín Ortego for all his suggestions and for his help with the genetic analyses programs to calculate relatedness. During this work E.G.V. was supported by a predoctoral fellowship from the Ministerio de Educación y Ciencia (MEC). This research was funded by projects GCL-2005-05611-C02-01 and 02/BOS (MICINN), POII10-0197-0167 (JCCM), PEII11-0161-4455 (JCCM), CGL2008-00095/BOS (MICINN) and 2005-SGR-00090 (Direcció General de Recerca de la Generalitat de Catalunya).

REFERENCES

Amos, W., Wilmer, J.W., Fullard, K., Burg, T.M., Croxall, J.P., Block, D. & Coulson, T. 2001. The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society of London Series B*, **268**: 2021–2027.

Aparicio, J. M., Cordero, P. J. & Veiga, J. P. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. *Animal Behaviour*, **62**: 1001-1006.

Aparicio, J. M., Ortego, J. & Cordero, P. J. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Molecular Ecology*, **15**: 4659-4665.

Berg, E. C. 2005. Parentage and reproductive success in the white-throated magpie-jay (*Calocitta formosa*), a cooperative breeder with female helpers. *Animal Behaviour*, **70**: 375-385..

Birkhead, T. R. & Møller, A. P. 1992. *Sperm Competition in Birds: Evolutionary Causes and Consequences*. London: Academic Press.

Bishop, J. M., O'Ryan, C. & Jarvis, J. U. M. 2007. Social common mole-rats enhance outbreeding via extra-pair mating. *Biology Letters*, **3**: 176-179.

Blomqvist, D., Andersson, M., Kupper, C., Cuthill, I. C., Kis, J., Lanctot, R. B. & Sandercock, B. K. 2002. Genetic similarity between mates and extra-pair parentage in three species of shorebirds. *Nature*, **419**: 613-615.

Blomqvist, D., Fessl, B., Hoi, H. & Kleindorfer, S. 2005. High frequency of extrapair fertilisations in the moustached warbler, a songbird with a variable breeding system. *Behaviour*, **142**: 1133-1148.

Brown, J. L. 1997. A theory of mate choice based on heterozygosity. *Behavioral Ecology*, **8**: 60–65.

Charmantier, A., Blondel, J., Perret, P. & Lambrechts, M. 2004. Do extra-pair paternities provide genetic benefits for female blue tits *Parus caeruleus*?. *Journal of Avian Biology*, **35**: 524–532.

Cordero, J. P, Viñuela, J., Aparicio, J. M. & Veiga, J. P. 2001. Seasonal variation in sex ratio and sexual egg dimorphism favouring daughters in first clutches of the spotless starling. *Journal of Evolutionary Biology*, **14**: 829-834.

Cordero, P. J., Veiga, J. P., Moreno, J. & Parkin, D. T. 2003. Extra-pair paternity in the nest of the facultatively polygynous spotless starling

(*Sturnus unicolor*). *Behavioral Ecology and Sociobiology*, **54**: 1–6.

Cordero, P. J., Aparicio, J. M. & Veiga, J. P. 2004. Parental genetic characteristics and hatching success in the spotless starling *Sturnus unicolor*. *Animal Behaviour*, **67**: 637-642.

Dawson, D. A., Hanotte, O., Greig, C., Stewart,I. R. K. & Burke, T. 2000. Polymorphic microsatellites in the Blue Tit *Parus caeruleus* and their cross-species utility in 20 songbird families. *Molecular Ecology*, **9**: 1941-1943.

Dreiss, A. N., Silva, N., Richard, M., Moyen, F., Théry, M., Møller, A. P. & Danchin, É. 2008. Condition-dependent genetic benefits of extrapair fertilization in female blue tits *Cyanistes caeruleus*. *Journal of Evolutionary Biology*, **21**: 1814-1822.

Doucet, S. M. 2002. Structural plumage coloration, male body size, and condition in the Blue-black Grassquit. *Condor*, **104**: 30-38.

Edler, R. & Frield, T. W. P. 2008. Within-pair young are more immunocompetent than extrapair young in mixed paternity broods of the red bishop. *Animal Behaviour*, **75**: 391-401.

Foerster, K., Delhey, K., Johnsen, A., Lifjeld, J. T. & Kempenaers, B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature*, **425**: 714-717.

Fossøy, F., Johnsen, A. & Lifjeld, J.T. 2008. Multiple genetic benefits of female promiscuity in a socially monogamous passerine. *Evolution*, **62**: 145-156.

Frasier, T. R. 2008. STORM: software for testing hypotheses of relatedness and mating patterns. *Molecular Ecology Resources*, **8**: 1263-1266.

Freeman-Gallant, C. R., Wheelwright, N. T., Meiklejohn, K. E. & Sollecito, S. V. 2006. Genetic similarity, extrapair paternity and offspring quality in Savannah sparrows (*Passerculus sandwichensis*). *Behavioral Ecology*, **17**: 952-958.

Friedl, T. W. P. & Klump, G. M. 2005. Extrapair fertilizations in red bishops (*Euplectes orix*): do females follow conditional extrapair strategies?. *Auk*, **122**: 57-70.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008a. Cross-amplified polymorphic microsatellites for the spotless starling *Sturnus unicolor*. *Ardeola*, **55**: 3-11.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008b. Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. *Animal Behaviour*, **76**: 345-353.

García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2009. Male feeding rate and extrapair paternity in the facultatively polygynous spotless starling. *Animal Behaviour*, **78**: 1335-1341.

Gebhardt-Henrich, S. G. & Richner, H. 1998. Causes of growth variation and its consequences for fitness. In: *Avian growth and development. Evolution within the altricial–precocial spectrum*. (Ed. by J.M. Starck & R.E. Ricklefs), pp 324-339. Oxford: Oxford University Press.

Gray, E. M. 1997. Female red-winged blackbirds accrue material benefits from copulating with extra-pair males. *Animal Behaviour*, **53**: 625-639.

Griffith, S. C., Owens, I. P. F. & Thuman, K. A. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**: 2195-2212.

Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. 1998. A DNA test to sex most birds. *Molecular Ecology*, **7**: 1071-1075.

Hagelin, J. C. & Ligon, J. D. 2001. Female quail prefer testosterone mediated traits , rather than the ornate plumage of males. *Animal Behaviour*, **61**: 465-476.

Hamilton, W. D. 1990. Mate choice near and far. *American Zoologist*, 30: 341-352.

Hansson, B., Bensch, S., Hasselquist, D & Åkesson, M. 2001. Microsatellite diversity predicts recruitment of sibling great reed warblers. *Proceedings of the Royal Society of London Series B*, **268**: 1287-1291.

Hasselquist, D., Bensch, S. & von Schantz, T. 1996. Correlation between male song repertoire, extrapair paternity and offspring survival in the great reed warbler. *Nature*, **381**: 229-232.

Jennions, M. D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews of the Cambridge Philosophical Society*, **75**: 21-64.

Kempenaers, B.& Dhondt, A. A. 1993. Why do females engage in extra-pair copulations? A review of hypotheses and their predictions. Belgian *Journal of Zoology*, **123**: 93-103.

Kempenaers, B., Verheyen, G. R., Van den Broeck, M., Burke, T., Van Broeckhoven, C. & Dhondt, A. A. 1992. Extrapair paternity results from female preferences for high quality males in the blue tit. *Nature*, **357**: 494-496.

Kempenaers, B., Geert, R. & Dhondt, A. 1997. Extrapair paternity in the blue tit (*Parus caeruleus*): female choice, male characteristics, and offspring quality. *Behavioral Ecology*, **8**: 481-492.

Kempenaers, B., Congdon, B., Boag, P. & Robertson, R. J. 1999. Extrapair paternity and egg hatchability in tree swallows: evidence for the genetic compatibility hypothesis?. *Behavioral Ecology*, **10**: 304-311.

Kokko, H. 2001. Fisherian and "good genes" benefits of mate choice: how (not) distinguish between them. *Ecology letters*, **4**: 322-326.

Krokene, C., Rigstad, K., Dale, M. & Lifjeld, J. T. 1998. The function of extrapair paternity in blue tits and great tits: good genes or fertility insurance? *Behavioural Ecology*, **9**: 649-656.

Krützen, M., Sherwin, W. B., Connor, R. C., Barré, L. M., Van de Casteele, T., Mann, J. & Brooks, R. 2003. Contrasting relatedness patterns in bottlenose dolphins (*Tursiops* sp.) with different alliance strategies. *Proceedings of the Royal Society of London Series B,* **270**: 497-502.

Leech, D. I., Hartley, I. R., Stewart, I. R. K., Griffith, S. C. & Burke, T. 2001. No effect of parental quality or extrapair paternity on brood sex ratio in the blue tit (*Parus caeruleus*). *Behavioral Ecology*, **12**: 674-680.

Li, C. C., Weeks, D. E., Chakravarti, A. 1993. Similarity of DNA fingerprints due to chance and relatedness. *Human Heredity*, **43**: 45-52.

Lynch, M. & Ritland, K. 1999. Estimation of pairwise relatedness with molecular markers. *Genetics*, **152**: 1753-1766.

Marshall, T. C., Slate, J., Kruuk, L. E. B. & Pemberton, J. M. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, 7: 639-655.

Marshall, R. C., Buchanan, K. L. & Catchpole, C. K. 2003. Sexual selection and individual genetic diversity in a songbird. *Proceedings of the Royal Society of London Series B,* Supplement, **2**: 248-250.

Masters, B. S., Hicks, B. G., Johnson, L. S. & Erb, L. A. 2003. Genotype and extra-pair paternity in the house wren: a rare-male effect?. *Proceedings of the Royal Society of London Series B*, **270**: 1393-1397.

McCarty, J. P. 2001. Variation in growth of nestling tree swallows across multiple temporal and spatial scales. *Auk*, **118**: 176-190.

McClelland, E. E., Penn, D. J. & Potts, W. K. 2003. Major histocompatibility complex heterozygote superiority during coinfection. *Infection and Immunity*, **71**: 2079-2086.

Møller, A. P. & Alatalo, R. V. 1999. Good-genes effects in sexual selection. *Proceedings of the Royal Society of London Series B*, **266**: 85-91.

Moreno, J., Veiga, J. P., Cordero, P. J. & Mínguez, E. 1999. Effects of paternal care on reproductive success in the polygynous spotless starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology*, **47**: 47–53..

O'Brien, E. L. & Dawson, R. D. 2007. Context-dependent genetic benefits of extra-pair mate choice in a socially monogamous passerine. *Behavioral Ecology and Sociobiology*, **61**: 775-782.

Ortego, J., Calabuig, G., Cordero, P. J. & Aparicio, J. M. 2007a. Egg production and individual genetic diversity in lesser kestrels. *Molecular Ecology*, **16**: 2383-2392.

Ortego, J., Aparicio, J. M., Calabuig, G. & Cordero, P. J.

2007b. Risk of ectoparasitism and genetic diversity in a wild lesser kestrel population. *Molecular Ecology*, **16:** 3712-3720.

Otter, K., Ratcliffe, L., Michaud, D. & Boag, P. T. 1998. Do female black-capped chickadees prefer high-ranking males as extrapair partners? *Behavioural Ecology and Sociobiology*, **43**: 25-36.

Pai, A. & Yan, G. 2002. Female mate choice in relation to heterozygosity in *Tribolium castaneum*. *Journal of Evolutionary Biology*, **15**: 1076-1082.

Petrie, M. & Kempenaers, B. 1998. Extra-pair paternity in birds: explaining variation between species and populations. *Trends in Ecology and Evolution,* **13**: 52-58.

Petrie, M., Doums, C. & Møller, A. P. 1998. The degree of extra-pair paternity increases with genetic variability. *Proceedings of the National Academy of Sciences of the United States of America*, **95**: 9390-9395.

Richardson, D. S., Jury, F. L., Dawson, D. A.,Salgueiro, P., Komdeur, J. & Burke, T. 2000. Fifty Seychelles Warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in *Sylviidae* species and their cross-species amplification in other passerine birds. *Molecular Ecology*, 9: 2226-2231.

Rosivall, B., Szöllösi, E., Hasselquist, D. & Török, J. 2009. Effects of extrapair paternity and sex on nestling growth and condition in the collared flycatcher *Ficedula albicollis*. *Animal Behaviour*, **77**: 611-617.

Rubenstein, D. R. 2005. Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*. *Molecular Ecology Notes*, **5**: 739-744.

Ryan, P. G. 2001. Morphological Heritability in a Hybrid Bunting Complex: Nesospiza at Inaccessible Island. *The Condor*, **103:** 429-438.

Saino, N., Primmer, C. R., Ellegren, H. & Møller, A. P. 1997. An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*). *Evolution*, **51**: 562-570.

Schmoll, T., Dietrich, V., Winkel, W., Epplen, J. T. & Lubjuhn, T. 2003. Long-term fitness consequences of female extrapair matings in a socially monogamous passerine. *Proceedings of the Royal Society of London Series B*, **270**: 259-264.

Schmoll, T., Dietrich, V., Winkel, W., Epplen, J. T., Schurr, F. & Lubjuhn, T. 2005. Paternal genetic effects on offspring fitness are context dependent within the extrapair mating system of a socially monogamous passerine. *Evolution*, **59**: 645-657.

Seddon, N., Amos, W. Mulder, R. A. & Tobias, J. A. 2004. Male heterozigosity predicts territory size, song structure and reproductive success in a cooperatively breeding bird. *Proceedings of the Royal Society of London Series B*, **271**: 1823-1829.

Sheldon, B. C. 1994. Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds, *Proceedings of the Royal Society of London Series B*, **257**: 25-30.

Sheldon, B. C., Merilä, J., Qvarnström, A., Gustafsson, L. & Ellegren, H. 1997. Paternal genetic contribution to offspring condition predicted by size of male secondary sexual character. *Proceedings of the Royal Society of London Series B*, **264**: 297-302.

Strohbach, S., Curio, E., Bathen, A., Epplen, J. T. & Lubjuhn, T. 1998. Extrapair paternity in the great tit (*Parus major*): a test of the 'good genes' hypothesis. *Behavioural Ecology*, **9**: 388-396.

Sundberg, J. & Dixon, A. 1996. Old, colourful male yellowhammers (*Emberiza citrinella*) benefit from extrapair copulations. *Animal Behaviour*, **52**: 113-122.

Suter, S.M., Keiser, M., Feignoux, R. & Meyer, D. R. 2007. Reed bunting females increase fitness through extra-pair mating with genetically dissimilar males. *Proceedings of the Royal Society of London Series B*, **274**: 2865-2871.

Tregenza, T. & Wedell, N. 2002. Polyandrous females avoid costs of inbreeding. *Nature*, **415**: 71-73.

Van De Casteele, T., Galbusera, P. & Matthysen, E. 2001. A comparison of microsatellite-based pairwise relatedness estimators. *Molecular Ecology*, **10**: 1539-1549.

Veiga, J. P., Moreno, J., Cordero, P. J. & Mínguez, E. 2001. Territory size and polygyny in the Spotless Starling: resource holding potencial or social inertia? *Canadian Journal of Zoology*, **79**: 1-6.

Veiga, J. P., Moreno, J., Arenas, M. & Sánchez, S. 2002. Reproductive consequences for males of paternal vs. territorial strategies in the polygynous spotless starling under variable ecological and social conditions. *Behavior*, **139**: 677-693.

Veiga, J. P., Viñuela, J., Cordero, P. J., Aparicio, J. M. & Polo, V. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. *Hormones and Behavior*, **46**: 47-53.

Wang, J. 2000. An estimator for pairwise relatedness using molecular markers. *Genetics*, **160**: 1203-1215.

Weatherhead, P. J. & Boag, P. T. 1995. Pair and extra-pair mating success relative to male quality in red-winged blackbirds. *Behavioral Ecology and Sociobiology*, **37**: 81-91.

Westneat, D. F. & Stewart, I. R. K. 2003. Extra-pair paternity in birds: causes, correlates and conflict. *Annual Review of Ecology Evolution and Systematics*, **34**: 365–396.

Westneat, D. F., Sherman, P. W. & Morton, M. L. 1990. The ecology and evolution of extrapair copulations in birds. *Current Ornithology*, **8**: 331-369.

Wetton J.H. & Parkin, D.T. 1991. An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. *Proceedings of the Royal Society series B*, **245**: 227-233.

Williams, G. C. 1975. *Sex and Evolution*. Princeton: Princeton University Press.

Yezerinac, S. M., Weatherhead, P. J. & Boag, P. T. 1996. Cuckoldry and lack of parentage-dependent paternal care in yellow warblers: a cost-benefit approach. *Animal Behaviour*, **52**: 821-832.

Zeh, J. A, & Zeh, D. W. 1996 The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London Series B*, **263**: 1711-1717.

Zeh, J. A. & Zeh, D. W. 1997. The evolution of polyandry II: postcopulatory defenses against genetic incompatibility. *Proceedings of the Royal Society of London Series B*, **264**: 69-75.

APPENDIX 1

ANCOVAs for testing the effect of the presence of EPO in the broods on clutch size, hatching success, nestling success, sex ratio, average offspring body mass, tarsus length and body condition. The results control for other influential variables.

Appendix Table 1. Response variable: Clutch size

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	11.678	1, 67	0.001
Year	1.349	5,67	0.255
Female treatment	2.003	1,67	0.162
Female heterozigosity (HL index)	0.630	1,67	0.430
Pair relatedness	0.868	1,67	0.355
Female body condition	0.172	1,67	0.680
Year*Presence of EPO in the brood	2.531	5,67	0.037
Female treatment*Presence of EPO in the brood	9.014	1,67	0.004

Appendix Table 2. Response variable: Hatching success

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.417	1,64	0.521
Year	1.952	5,64	0.098
Female treatment	0.341	1,64	0.561
Female heterozigosity (HL index)	1.068	1,64	0.305
Pair relatedness	0.558	1,64	0.458
Female body condition	0.251	1,64	0.618
Male status	3.137	1,64	0.081
Clutch size	11.017	1,64	0.001

Appendix Table 3. Response variable: Nestling success

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.503	1,42	0.482
Year	3.049	2,42	0.058
Female treatment	0.138	1,42	0.712
Female heterozigosity (HL index)	0.738	1,42	0.395
Pair relatedness	0.246	1,42	0.622
Number of nestlings	2.338	1,42	0.134
Parental feeding rate	6.694	1,42	0.013

Appendix Table 4. Response variable: Sex ratio

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.168	1,38	0.684
Year	1.566	5,38	0.193
Female treatment	0.997	1,38	0.324
Female heterozigosity (HL index)	0.664	1,38	0.420
Pair relatedness	0.182	1,38	0.672
Number of fledglings	0.697	1,38	0.409

Appendix Table 5. Response variable: Offspring body mass at 15 days

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.276	1,40	0.602
Year	3.657	2,40	0.035
Female treatment	3,136	1,40	0.084
Female heterozigosity (HL index)	1.056	1,40	0.310
Pair relatedness	0.817	1,40	0.371
Parental feeding rate	0.889	1,40	0.351
Number of fledglings	15.064	1,40	<0.001

Appendix Table 6. Response variable: Offspring tarsus length at 15 days

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.304	1,40	0.584
Year	5.803	2,40	0.006
Female treatment	0.538	1,40	0.468
Female heterozigosity (HL index)	0.666	1,40	0.419
Pair relatedness	0.713	1,40	0.403
Parental feeding rate	5.683	1,40	0.022
Number of fledglings	0.834	1,40	0.367

Appendix Table 7. Response variables: Offspring body condition at 15 days

Variable	*F*	g.l.	*P*
Presence of EPO in the brood	0.398	1,40	0.532
Year	0.613	2,40	0.547
Female treatment	1.256	1,40	0.269
Female heterozigosity (HL index)	0.146	1,40	0.704
Pair relatedness	1.427	1,40	0.239
Parental feeding rate	0.410	1,40	0.526
Number of fledglings	19.394	1,40	<0.001

DISCUSIÓN GENERAL

El estudio de los sistemas de apareamiento y las estrategias reproductoras de los individuos de una especie es una tarea muy compleja debido a los diferentes factores que influyen sobre ellas y a que diferentes estrategias reproductoras pueden estar operando de forma simultánea. Así, aunque tradicionalmente este tipo de estudios se basaba únicamente en la observación de las conductas de los individuos, las evidencias a lo largo de las últimas décadas nos indican que, si bien los datos observacionales son cruciales, estos deben ir siempre acompañados por estudios de parentesco, puesto que las relaciones sociales y genéticas entre individuos no siempre son coincidentes y pueden proporcionar una imagen desvirtuada de sus verdaderas estrategias reproductoras. Ello ha llevado al desarrollo de numerosas metodologías para analizar el parentesco genético entre individuos, entre las que destacan los microsatélites por sus múltiples ventajas frente a otros marcadores moleculares (Ver Metodología General y Capítulo 1).

En esta tesis, conscientes de tal necesidad, se proporcionan 6 cebadores microsatélite polimórficos de amplificación cruzada y se comprueba su utilidad en estudios genéticos, en concreto para la identificación de individuos y para el análisis de parentesco (Ver Capítulo 1). La optimización de estos loci microsatélite de amplificación cruzada constituye la base para los demás estudios llevados a lo largo del presente trabajo y amplia el panel de loci disponibles, que pueden ser usados para estudios de diversidad genética, dispersión, estructura de la población y correlatos del éxito de emparejamiento y reproductor en el estornino negro. Así, el empleo de los loci microsatélite optimizados permite corroborar la existencia de la paternidad extra-pareja como un fenómeno frecuente en esta especie, y también la presencia de casos de parasitismo y pseudoparasitismo. A pesar de que la ocurrencia de estas estrategias era conocida con anterioridad a la realización de nuestro trabajo en esta y otras especies poligínicas, el papel que cada sexo, y en especial que la hembra, puede desempeñar en ellas no estaba del todo claro. La presente tesis doctoral pone de manifiesto sin ambigüedad que las hembras de estornino negro tienen un papel activo en el sistema promiscuo del estornino negro, y que su fenotipo, al menos en lo que respecta a edad, longitud de los ornamentos y niveles de testosterona circulante en sangre, resulta clave para explicar las tasas de paternidad extra-pareja encontradas en sus nidos.

En este sentido, la manipulación hormonal llevada a cabo en el Capítulo 2 permite demostrar por primera vez en aves que los niveles de testosterona circulante en las hembras incrementados experimentalmente tienen como consecuencia una reducción significativa de la paternidad extra-pareja en sus nidos en comparación con las hembras no tratadas. La menor presencia de pollos extra-pareja (EPOs) en las polladas de las hembras tratadas con testosterona no puede achacarse a diferencias de mortalidad entre EPO y pollos intra-pareja (WPO) y tampoco concuerda con los resultados encontrados en machos en el sentido de que la testosterona mejora el éxito de emparejamiento de los individuos, al menos en lo que a obtención de fertilizaciones adicionales por parte de las hembras se refiere. Estos resultados pueden deberse a que la testosterona provoca cambios fisiológicos, de status o de comportamiento a largo plazo en las hembras que las hacen menos atractivas para los machos (las masculiniza), las vuelven menos proclives a entablar cópulas extra-pareja (EPCs) o más capaces de resistirlas, mas agresivas o incluso pueden hacerlas adoptar conductas propias de hembras de edad superior. Sin embargo, conocer con detalle cuáles son los cambios producidos por la hormona necesitaría más investigación. Sería de interés llevar a cabo estudios observacionales más precisos en el estornino negro y más experimentos en diferentes especies de aves u otros vertebrados con sistemas de apareamiento distintos a la poliginia facultativa para poder generalizar los efectos de la testosterona puestos de manifiesto en esta tesis sobre las estrategias de emparejamiento de las hembras. Los resultados obtenidos en el Capítulo 2 no son concluyentes para afirmar cuál es el sexo que inicia o busca las EPCs en el estornino negro, pero sí lo son para determinar que las hembras son parte activa en la toma de esta decisión, ya sea iniciando las EPCs o evitándolas, afirmación que también sustentan los resultados obtenidos en el Capítulo 4. En concreto, en dicho capítulo se encontró que tanto la pérdida de paternidad de los machos en sus nidos como la ganancia de paternidad en nidos ajenos estaba relacionada con las características (en este caso fenotípicas) de sus hembras sociales. Así, los machos emparejados con hembras mas maduras y ornamentadas perdieron menos paternidad en sus nidos y ganaron más paternidad en nidos ajenos, resultado este que vuelve a incidir en el importante papel que desempeñan las hembras en el desarrollo y mantenimiento de las estrategias extra-pareja en esta especie.

La presencia de EPOs en un nido no implica necesariamente que exista un coste de poligínia para los machos, ya que tanto los monógamos como los polígamos tienen EPOs en sus nidos. Por el contrario, la paternidad extra-pareja parece suponer por sí misma una estrategia reproductora alternativa que puede reportar beneficios tanto para los machos como para las hembras. Los beneficios para los machos de aparearse con múltiples hembras simultáneamente, ya sea de forma social (poliginia) o mediante emparejamientos extra-pareja resultan evidentes ya que esto les permite aumentar, al menos aparentemente, su éxito reproductor. Así, los resultados obtenidos en el Capítulo 4 indican que el éxito reproductor del macho (entendido como el número de hembras sociales que éste es capaz de atraer y que inician una puesta en sus nidos) está asociado positivamente con su tamaño corporal e independientemente también con su edad y ornamentos, variables estas dos últimas que están intrínsecamente ligadas y son probablemente indicadoras de la calidad del individuo. De esta forma los machos más grandes, maduros y ornamentados serían los que tendrían un mayor éxito reproductor, probablemente porque por su tamaño y experiencia pueden ofrecer una mejor defensa de los nidos y los pollos frente a otros machos y quizás frente a depredadores, mientras que sus ornamentos y probada supervivencia podrían indicar una mayor calidad (genética y/o fenotípica). Sin embargo, los resultados encontrados en ese mismo capítulo y de forma más concluyente en el Capítulo 5 indican que estas características (tamaño, edad y longitud de los ornamentos) no estarían siendo seleccionadas por las hembras que buscan machos extra-pareja. De hecho, en el Capítulo 4 se encontró que las características fenotípicas del macho (morfología y edad) no se asocian a una mayor ganancia de paternidad en nidos ajenos o a una mayor pérdida de paternidad en los propios, por lo que podemos deducir que no influyen sobre las conductas promiscuas en esta especie, asumiendo como conductas promiscuas aquellas que suponen fertilizaciones extra-pareja. En el Capítulo 5, además de corroborar este resultado de que los machos promiscuos no presentan ninguna característica particular que los diferencie de los machos no promiscuos, tampoco se encontraron diferencias significativas en ninguno de los caracteres analizados mediante tests apareados al comparar los machos sociales y extra-pareja de una misma hembra, al menos hasta donde podemos averiguar con las variables usadas.

Estos resultados sugieren de forma general que las hembras de estornino negro no buscan o aceptan EPCs con machos de fenotipo tipíficado en cuanto a edad y morfología con el fin de mejorar la calidad genética de sus descendientes a través de la herencia de sus caracteres. Es decir, en el estornino negro no existe un standard general de macho extra-pareja, sino que existe una gran variabilidad en las características de los machos identificados como padres genéticos. Todo ello sugiere que la hipótesis de los "buenos genes" (Ver Capítulo 4 y 5) podría explicar, al menos parcialmente, el mantenimiento de la poliginia en esta especie (aunque esta afirmación necesita un soporte empírico más sólido) pero no el mantenimiento de los emparejamientos extra-pareja. La hipótesis de los "buenos genes" es una de las hipótesis mas estudiadas y aceptadas para explicar el mantenimiento de la paternidad extra-pareja en aves pero nuestros resultados sugieren que no constituye una explicación convincente en el estornino negro.

Los resultados expuestos en los Capítulos 4 y 5 de esta tesis abajo listados:

- El número habitual de EPOs por pollada fue de uno y en el caso de existir más, estos fueron asignados a distintos padres genéticos.
- No se encontraron diferencias fenotípicas ni de supervivencia entre ausencia de diferencias entre los EPOs y WPOs de una misma pollada.
- Se encontraron EPOs en, al menos, el 24% de las polladas estudiadas en cada estación reproductora.

- Los machos que ganaron paternidad en otros nidos no incrementaron su éxito reproductor, ya que también perdieron paternidad en sus propios nidos.
- Las polladas con EPO fueron aquellas cuyos padres tenían una mayor similitud genética entre ambos.
- No se encontraron diferencias entre los machos sociales y extra-pareja.

sugieren que una de las hipótesis más plausibles para explicar la paternidad extra-pareja como estrategia reproductora alternativa de las hembras de estornino negro, es la hipótesis de la diversidad genética. Según la cual las hembras de estornino negro podrían buscar o aceptar copular con machos extra-pareja con el fin de aumentar la variabilidad genética de sus descendencia y por lo tanto su viabilidad. De hecho la mayoría de sus predicciones, listadas a continuacion, se cumplen en nuestro estudio:

- Pocos EPOs por pollada, o si hay muchos, deberían pertenecer a diferentes machos.
- Ausencia de diferencias entre los EPOs y los WPOs.
- La mayoría de hembras de la población llevarían a cabo EPCs.
- La varianza en el éxito reproductivo del macho no debería incrementarse a causa de las EPCs.
- La presencia de EPOs en un nido debería ser más común si los miembros de la pareja están relacionados o son similares genéticamente.
- Las hembras no serían capaces de evaluar la calidad de los machos extra-pareja.

y aquellas que no lo hacen, como por ejemplo que no encontrásemos diferencias significativas entre las polladas con paternidad mixta y las polladas sin EPOs, pueden ser explicadas por la dificultad de demostrar efectos genéticos, ya sea por su débil magnitud o por carecer de la potencia estadística suficiente para ello.

Otras hipótesis que abogan por el mantenimiento de la paternidad extra-pareja como estrategia para la obtención de beneficios genéticos por parte de las hembras son las hipótesis de compatibilidad genética y de garantía de la fertilidad. Aunque algunos resultados pueden ajustarse a sus predicciones, como la ausencia de diferencias entre machos sociales y extra-pareja, no ocurre así en la mayoría de los casos. Así por ejemplo, en lo que respecta a la hipótesis de compatibilidad genética no se detectaron diferencias significativas en la heterocigosidad de los EPOs frente a los WPOs. Por su parte, en lo que respeta a la hipótesis de garantía de la fertilidad no hubo significativamente más EPOs en los nidos de los machos poligínicos ni un mayor éxito de eclosión en las polladas con EPOs. Estos resultados llevan a pensar que estas hipótesis pueden ser descartadas como posibles explicaciones para el mantenimiento de la paternidad extra-pareja en el estornino negro. Lo mismo ocurre con aquellas que intentan explicar el mantenimiento de esta estrategia reproductora con el fin de obtener beneficios directos por parte de las hembras, ya que los machos extra-pareja identificados no parecen colaborar en la defensa de los nidos ni en la ceba de los pollos, aunque mas investigación es necesaria en este sentido.

Los resultados mostrados principalmente a lo largo de los Capítulos 2, 4 y 5 de esta tesis doctoral sugieren de forma general que las hembras salen beneficiadas del mantenimiento de la paternidad extra-pareja como estrategia reproductora alternativa, a pesar de los costes implícitos en la misma. Uno de los principales costes para las hembras derivados de esta estrategia, y que ha sido mostrado ya en numerosos estudios, es la reducción del cuidado parental de los machos hacia los pollos en aquellos nidos en los que el macho no tiene certeza de su propia paternidad. Los resultados obtenidos en el Capítulo 3 muestran que en el estornino negro este coste no parece ser demasiado importante, ya que el cuidado parental ejercido por el macho, aunque es importante en las primeras fases de desarrollo de los pollos, no resulta ser crucial para su supervivencia final y que la ausencia de cuidado parental por parte del macho parece poder ser compensada por la hembras. Además, la ausencia de relación entre la paternidad y la reducción del cuidado parental del macho en el estornino negro se ajusta a lo establecido en los modelos teóricos para las especies con poliginia facultativa, en las que la presencia de EPO no varía de acuerdo a un patrón definido entre intentos reproductores o edades del mismo macho y la existencia de claves fiables que le permitan al macho evaluar su grado de paternidad en la pollada es poco probable.

Un resultado sorprendente y novedoso es que los machos que pierden paternidad en sus nidos no sólo no ceban menos a sus pollos, tal y como era esperable, sino que ceban más. No obstante, se necesita más investigación para consolidar esta conclusión. En cualquier caso, este resultado puede ser explicado de acuerdo a varias hipótesis: en primer lugar, la inversión parental del macho puede constituir una estregia para mantener el vínculo con la misma hembra en las siguientes estaciones de cría, lo cual es propio de especies de vida relativamente larga como el estornino negro. En segundo lugar, es posible que la elevada tasa de ceba de los machos sociales en polladas con EPOs se deba a un mayor valor intrínseco de estas polladas, un resultado que estaría en concordancia con los obtenidos en el Capítulo 5, según los cuales el beneficio fundamental del mantenimiento de la paternidad extra-pareja para las hembras es la obtención de polladas mas viables. En tercer lugar, la mayor inversión paternal en polladas con EPOs podría explicarse desde el punto de vista de los pollos, ya que el aumento de la frecuencia de ceba en las polladas con paternidad mixta podrían responder a un incremento en los comportamientos de petición de alimento ("begging") en estas polladas (Ver Capítulo 3) respecto a las polladas con un solo padre genético. En el primer caso deberíamos comprobar si aquellos machos con tasas de ceba mas bajas tienen una mayor tasa de divorcio o menor éxito en la obtención de pareja en periodos de reproduccion posteriores, mientras que la segunda hipótesis podría ser abordada mediante estudios de reclutamiento de los pollos para ver si hay diferencias de supervivencia entre los pertenecientes a polladas de paternidad mixta y los criados en polladas con un único padre genético. Finalmente desde el punto de vista de los pollos, estudios que relacionen el nivel de "begging" y de parentesco en las polladas se hacen necesarios para esclarecer esta cuestión.

En conclusión, el hecho de que el fenotipo de las hembras de estornino negro esté significativamente relacionado con la tasa de paternidad extra-pareja detectada en sus nidos (Ver Capítulos 2 y 4) sugiere que las hembras no son sólo sujetos pasivos en el desarrollo de las estrategias promiscuas, y de manera particular en la ocurrencia de emparejamientos y fertilizaciones extra-pareja, sino que pueden obtener beneficios genéticos de ellas. En concreto una mayor viabilidad de sus polladas es el beneficio más probable tal y como propone la hipótesis de diversidad genética (Ver Capítulo 5). Además, los resultados obtenidos en los Capítulos 3 y 4 sugieren que, para los machos los beneficios de la paternidad extra-pareja no parecen tener una gran repercusión sobre su eficacia biológica en comparación con los que les reporta la poliginia; y que los supuestos costes para las hembras derivados de la conductas promiscuas, tal como la reducción en la tasa de ceba a los pollos por parte del macho, no son severos en el caso de especies con estrategias sociales, demográficas y reproductoras similares a las del estornino negro (Capítulo 3).

CONCLUSIONES

1) En este trabajo, hemos demostrado por primera vez que la administración exógena de testosterona en hembras de aves reduce significativamente la proporción de pollos extra-pareja en sus nidos respecto al lote de hembras controles. En el estornino negro, el incremento de testosterona en las hembras podría reducir su atractivo para potenciales machos extra-pareja o facilitar a las hembras el rechazo de machos no deseados

2) La promiscuidad de la hembra, traducida como la proporción de pollos extra-pareja en sus nidos, disminuye con la edad, efecto que es independiente del tratamiento hormonal. La edad, ya sea por experiencia o por constricciones fisiológicas las hace menos proclives a conductas promiscuas por las mismas razones señaladas en el punto anterior.

3) En contra de la predicción de modelos teóricos, los machos de estornino negro no reducen su tasa de ceba a los pollos con el aumento de la proporción de pollos extra-pareja en la pollada. Este resultado puede explicarse por la naturaleza poligínica de esta especie, en la que el éxito reproductor de los machos depende principalmente de su grado de poligamia, por el hecho de que la ocurrencia de paternidad extra-pareja es impredecible y por la aparente falta de claves fiables para los machos para reconocer a sus verdaderos hijos genéticos frente a los pollos extra-pareja en la pollada.

4) Características morfológicas de los machos tales como la longitud de las plumas del pecho y el tamaño corporal se asocian positivamente y de forma independiente al número de parejas sociales que estos consiguen y por tanto a su éxito reproductor general, ya que los machos poligínicos no pierden más paternidad que los machos monógamos.

5) Ni la pérdida de paternidad del macho dentro de su propio nido, ni la ganancia de paternidad en nidos ajenos están asociadas con las características fenotípicas de éstos. Si asumimos el fenotipo del individuo como reflejo de una calidad genética subyacente, estos resultados indican que la promiscuidad en el estornino

negro no está relacionada con la búsqueda de individuos de mayor calidad genética que la pareja social, o lo que es lo mismo, los resultados no apoyan la hipótesis de los "buenos genes".

6) Tanto la pérdida de paternidad del macho dentro de su propio nido, como su ganancia en nidos ajenos está asociada con las características de su pareja social. Machos emparejados con hembras maduras pierden menos paternidad en sus nidos y ganan más paternidad en nidos ajenos. Los machos apareados con hembras maduras podrían invertir menos tiempo en guardarlas y más en la búsqueda de otras hembras con las que copular para aumentar así su éxito reproductor.

7) Los patrones de paternidad muestran que los machos sociales y los machos extra-pareja no presentan diferencias fenotípicas entre ellos. Así mismo, no existen diferencias entre pollos intra y extra-pareja del mismo nido en las variables fenotípicas consideradas, lo que sugiere la ausencia de selección fenotípica o de transmisión de caracteres indicadores de calidad de padres a hijos, en contra de la hipótesis de los "buenos genes".

8) A mayor similitud genética de las parejas sociales, mayor probabilidad de aparición de pollos extra-pareja en las polladas. Este resultado junto con los patrones de paternidad encontrados en esta especie sugiere que, de entre todas las hipótesis analizadas, la hipótesis de diversidad genética es la más plausible para explicar el mantenimiento de la paternidad extrapareja en el estornino negro desde el punto de vista de las hembras.