UNIVERSIDAD NACIONAL AUTÓNOMA

DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE ECOLOGÍA

SELECCIÓN SEXUAL E INVERSIÓN DIFERENCIAL EN EL BOBO DE PATAS AZULES, *Sula nebouxii*

TESIS

QUE PARA OBTENER EL GRADO ACADÉMICO DE

DOCTOR EN CIENCIAS

P R E S E N T A

FABRICE DENTRESSANGLE

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Junio 2009

A mi familia

AGRADECIMIENTOS

Al Doctorado en Ciencias Biomédicas, UNAM.

Al Instituto de Ecología de la UNAM, especialmente al Laboratorio de Conducta Animal, al Consejo Nacional de Ciencia y Tecnología, CONACyT, y a la Dirección General de Estudios de Posgrado, UNAM quienes financiaron esta tesis.

A la SEMARNAT, al Parque Nacional de la Isla Isabel y la Armada de México por los permisos y el apoyo logístico

A mi Tutora,

Dra. Roxana Torres por el apoyo, asesoría y las horas invertidas en la revisión, análisis y discusión tanto del diseño experimental como de la interpretación de los resultados de esta investigación. Gracias por su paciencia y su participación en la consolidación de mi formación académica y por este trabajo de campo que nunca olvidare.

Al Comité Tutoral,

Dr. Alejandro Córdoba Aguilar y Dr. Marco Cerbón Cervantes, agradezco infinitamente el apoyo y la asesoría en cada una de las fases de la tesis, en especial en aquellas en las que fueron críticas para llevar a buen término este proyecto.

Al Laboratorio de Hormonas Esteroides del Instituto Nacional de Nutrición.

Especialmente a Lourdes Boeck y Roberto Chavira para su ayuda preciosa en el montaje de la técnica de análisis de los andrógenos, gracias por su simpatía y buen humor, y muchas gracias por haberme considerado como parte de su equipo.

Al Jurado del Examen de Candidatura,

Dr. Hugh Drummond Durey

Dra. Carolina Valdespino Quevedo
Dr. Carlos Valverde Rodríguez
Dra. Robyn Hudson
Dr. Alejandro Cordoba Aguilar

A todos gracias por sus comentarios y criticas constructivas durante el examen

Al Jurado del Examen de Grado,
Dr. Hugh Drummond Durey
Dra. Carolina Valdespino Quevedo
Dr. Carlos Valverde Rodríguez
Dr. Jorge Ernesto Schondube Frieldwold
Dr. Marco Cerbon Cervantes

Gracias a todos por los comentarios y sugerencias al documento final de la tesis.

Agradecimientos personales

A Alejandro González, quien me dio la oportunidad de conocer México, de participar en su trabajo de campo en la Mancha y de descubrir la investigación que se realiza en el laboratorio de conducta animal. Igualmente por su ayuda sobre los manuscritos y sobre todo como amigo. Por haber pasado momentos inolvidables en la excelente compañía de Ramadan y Arturo.

A todos los que me ayudaron en el trabajo de campo de miles de maneras diferentes, por su buen humor y su apoyo, Emma Villaseñor, Daniel González, Cristina Carmona (Momo), Natalia Lifshitz, Judith Morales, Rene Beamonte, Genoveva Trejo, Alejandra Ramos, Oscar Sánchez, Myrna Hernandez, Adriana Nava.

A todos los pescadores de la isla Isabel, especialmente a Yamaha y su familia, y los Poli's por su apoyo logístico y sobre todo su apoyo moral. Por haber pasado momentos inolvidables en esta isla maravillosa, para llevarme a pescar y enseñarme a tomar la vida por el buen lado y por todas estas risas….

A todos mis amigos y compañeros, Alejandro González Voyer, Sin-Yeon Kim, Alejandro Ríos Chelén, César González, Omar Arellano, Adriana Vallarino, Monica Pimienta, Oscar Sánchez, Cristina Carmona, Natalia Lifshitz, Ruth Rodríguez, Irene Barbosa, René Beamonte, Edgar aviles, Sergio Ancona, Ireri Brumón, Eira Bermúdez, Alejandra Valero, Dr. Hugh Drummond y Dr Constantino Macias, y a todos los *Lunáticos*.

Un agradecimiento muy especial a todos los que estuvieron conmigo a lo largo de estos años pasado en México, en los buenos momentos como en los malos, Merci du fond du coeur à Ramadan Salazar, Daniel Gonzalez y Pablo Gesundheit.

A mi familia
Quienes siempre me apoyaron en mis decisiones, me motivaron en los momentos difíciles y gracias por su apoyo financiero que me permitió terminar esta tesis.
Gracias a mi abuelo por haberme dado este gusto de la naturaleza y esta pasión por las civilizaciones antiguas que conocí de más cerca viviendo en México.

A mis amigos los Franchutes
Noé, Jérome, Erwin, Blandine, Bricoul, Nono, y toda la banda de Vanosc, mi pueblito querido, quienes siempre estuvieron cerca a pesar de la distancia.

Finalmente y sobre todo, a los bobos de patas azules por ser tan simpáticos y carismáticos.

CONTENIDO

Resumen

Los efectos maternos son reconocidos como un mecanismo adaptativo por el cual las hembras modifican su inversión en las crías de acuerdo a variables que pueden influir sobre la adecuación de la descendencia en un contexto específico. Por ejemplo, las hembras pueden modular su inversión en términos del tamaño del huevo y/o su composición bioquímica de acuerdo a variables que reflejen las futuras condiciones de crianza. Particularmente, cuando los ornamentos indican la calidad o la condición del macho, las hembras podrían invertir en su reproducción en función del atractivo de su pareja. En el bobo de patas azules, el color de las patas de los machos es una señal bajo selección sexual; se trata de una señal dinámica, dependiente de la condición nutricional y que esta relacionada con el esfuerzo paternal. Los machos participan en la incubación y el cuidado de las crías y un estudio experimental ha mostrado que una disminución en la condición del macho durante el periodo de cuidado parental tiene un efecto negativo en la condición de la hembra. Por lo tanto, las hembras deberían ajustar su inversión en la reproducción en función de la condición del macho. De acuerdo con lo anterior, un estudio previo encontró que cuando se modificó experimentalmente el color de las patas del macho hacia un azul oscuro, simulando un macho en baja condición, las hembras redujeron el volumen de los huevos. Este resultado sugiere que las hembras podrían estar favoreciendo la reducción de la nidada cuando las expectativas de éxito reproductivo son bajas. En este estudio evaluamos si las condiciones ambientales y el color de las patas de los machos influyen en la inversión materna en los huevos (tamaño de huevo y contenido de andrógenos) y si esta inversión diferencial afecta el éxito de eclosión, la respuesta inmune y las habilidades competitivas de las crías (solicitud de alimento y agresión). Para cumplir con los objetivos, modificamos el color de las patas hacia un azul más oscuro 24h después de la puesta del primer huevo y registramos la inversión de la hembra en términos de masa, volumen y concentraciones de andrógenos (androstenediona, A4,

testosterona, T, y dihidrotestosterona, DHT) de los segundos huevos. En el grupo control se llevaron a cabo la misma manipulación y registros pero no se modificó el color de patas. Para evaluar los efectos de esta inversión diferencial en los huevos, comparamos el éxito de eclosión y las concentraciones plasmáticas de andrógenos de las crías a la edad de 7 días, el crecimiento y la respuesta inmune celular a los 15 días entre nidos experimentales y controles. Adicionalmente, comparamos las conductas de solicitud de alimento y de agresión entre nidos controles y experimentales cuando las segundas crías tenían 10 días. Encontramos que relativo al primer huevo de la puesta, las hembras pusieron segundos huevos más pesados durante un año de El Niño (condiciones ambientales pobres) que durante un año bueno. Las hembras apareadas con un macho experimental redujeron el volumen y la masa del segundo huevo y pospusieron la puesta del segundo huevo en comparación con hembras del grupo control. La concentración absoluta de A4 (pero no de T) en los segundos huevos fue mayor durante un año con condiciones pobres que en un año bueno. Solo durante el año de El Niño, las hembras redujeron las concentraciones relativas de A4 en el vitelo (pero no de T) cuando estuvieron apareadas con un macho experimental. Los segundos huevos del grupo experimental tuvieron un éxito de eclosión menor y fueron fertilizados en una menor proporción comparado a los segundos huevos del grupo control. Las crías experimentales (que tuvieron un padre al cual se modificó el color de patas a un azul oscuro) tuvieron concentraciones de A4 y DHT y respuesta inmune celular menores a las crías controles, sugiriendo en el caso de la A4 una inversión menor de esta hormona en el vitelo (la concentración de A4 en el vitelo y en el plasma de las crías se correlacionan positivamente). Las crías experimentales pasaron en promedio 6.6% menos tiempo solicitando alimento que las crías controles, pero no detectamos diferencias en la tasa de crecimiento. Las conductas agresivas fueron 2.5 veces mas frecuentes en las crías experimentales que en las controles, y estuvieron positivamente correlacionadas con la concentración plasmática de A4; además las

crías hembras fueron 4 veces mas agresivas que los machos. Los resultados sugieren que las hembras de bobo de patas azules pueden ajustar su inversión en el tamaño de los huevos y su contenido en andrógenos de acuerdo a las condiciones ambientales y al color de las patas de su macho durante la puesta. La reducción en el éxito de eclosión, la respuesta inmune celular y la modificación en las conductas de solicitud de alimento y agresión de las crías sugieren que las hembras favorecen la reducción de la nidada cuando la condición aparente del macho, indicada por el color de sus patas, se deteriora.

Abstract

Maternal effects are recognized as an adaptive mechanism by which females vary investment in offspring according to variables that may influence the offspring fitness in a particular context. For example, females may modulate their investment in egg size and/or egg composition according to variables that are susceptible to reflect future rearing conditions. Particularly, when sexual ornaments signal the quality or condition of the male, females may invest in reproduction as a function of the attractiveness of their mate. In the blue-footed booby, male feet colour is a dynamic condition-dependent sexually selected trait that is related to paternal effort. Paternal contribution during rearing is a key factor for female breeding success, and a previous study found that when male feet colour was modified to a darker blue, simulating a male in poor condition, females reduced egg volume suggesting that females may favor brood reduction when breeding success expectation is poor. In this study, we tested whether breeding conditions and male feet colour influence blue-footed booby female investment in eggs (egg size and yolk androgens content) and whether this differential investment in eggs affects hatching success and chicks' immune response and competitive abilities (begging and aggressive behavior). To achieve our aims, we experimentally reduced male attractiveness by modifying male feet colour to a darker blue within 24 hrs after the first egg was laid and recorded female investment in the second egg mass, volume, and yolk androgen concentrations (androstenedione, A4, testosterone, T, and dihydrotestosterone, DHT). To evaluate the effects of differential investment in eggs according to male feet colour, we compared hatching success and chicks´ plasma androgen concentrations at the age of 7-d, growth rate and cellular immune response at the age of 15-d from control and experimental nests. Additionally, we compared begging and aggressive behavior of 10-d old second chicks from control and experimental nests. We found that, relative to the first egg in the clutch,

females laid heavier second eggs during an “El Niño” year (poor breeding conditions) than during a good year. Females paired with males with duller feet colour reduced second-egg mass and volume and delayed the laying of the second egg. Absolute yolk A4 concentration (but not T) in second eggs was higher during a poor year than during a good year. Only during a year with poor breeding conditions, females paired with experimental males decreased the relative A4 concentration (but not T) in the second egg compared to control females. Second eggs in the experimental group had lower hatching success and were fertilized in a lower proportion than in the control group. Second chicks fathered by males with duller feet had lower A4 and DHT plasma concentrations and lower cellular immune response compared to second control chicks, suggesting in the case of A4 a lower investment of this androgen in the yolk. Chicks hatched from eggs fathered by experimental males spent 6.6% less time begging than controls, but no differences in growth rate were found. Aggression was 2.5 fold higher in experimental than control chicks, and was positively related to plasma A4 concentration; moreover, female chicks were 4 times more aggressive than males. Hence, the results suggest that blue-footed booby females are able to adjust their investment in egg size and yolk androgens according to environmental conditions and male feet colour at laying. The reduction of hatching success, weakening of chicks’ immune response and modification of chicks´ begging and aggressive behavior suggests that females may favor brood reduction when the mate apparent condition, indicated by feet color, deteriorates.

SELECCIÓN SEXUAL E INVERSIÓN DIFERENCIAL EN EL BOBO DE PATAS AZULES, *Sula nebouxii*

INTRODUCCION

En la última década, los estudios genéticos así como los estudios ecológicos han revelado que el ambiente experimentado por las "madres" puede tener una fuerte influencia en el fenotipo de sus crías (Wolf et al. 1998; Pigliucci 2005; Mousseau y Fox 1998a, b; Price 1998; Qvarnström y Price 2001). Esta influencia materna, llamada efectos maternos, ocurre cuando el fenotipo de un individuo no esta solamente determinado por su propio genotipo y las condiciones ambientales que experimenta durante el desarrollo, sino también por el fenotipo o el ambiente de su madre (Wade 1998). Las condiciones experimentadas por las hembras a lo largo de su vida pueden llevar a variaciones en su tasa de crecimiento, su condición corporal o su estado fisiológico lo cual influirá en el fenotipo de sus crías por ejemplo, vía transmisión de factores citoplásmicos o deposición de hormonas (Mousseau y Fox 1998; Badyaev et al. 2002). De la misma manera, la conducta de la hembra, como el momento y el lugar donde se reproduce, el número de crías que produce así como las diferencias competitivas entre las crías, pueden tener implicaciones a nivel de la competencia entre hermanos o entre conespecíficos durante la etapa de desarrollo temprano que podrían tener consecuencias importantes sobre la adecuación de la progenie (Wade 1998; Lack 1954). Los efectos maternos se reconocen ahora como un mecanismo adaptativo mediante el cual el ambiente experimentado por las hembras a lo largo de su vida se "transmite" a su progenie y resulta en la generación de variación fenotípica en las crías (Mousseau y Fox 1998a, b).

Los efectos maternos se han definido como la regresión parcial que existe entre el fenotipo de las crías y el fenotipo de la hembra (Kirkpatrick y Land 1989). Lo anterior incluye

los efectos producidos por la herencia genética de la hembra, es decir el grado de similitud entre la hembra y sus crías en algunos caracteres fenotípicos, así como los efectos por selección materna producidos por todas las acciones realizadas por la hembra que influyan directamente sobre la adecuación de las crías (Kirkpatrick y Lande 1989), y por lo tanto en la adecuación total de las hembras (Mousseau y Fox 1998a y b). De acuerdo a lo anterior se ha documentado que los efectos maternos se modulan en función de parámetros que reflejan las condiciones futuras de crianza como por ejemplo factores ambientales (Mousseau y Fox 1998a y b; Badyaev et al. 2002; Gil 2003; Gil et al. 2004a), la densidad de anidación (Mazuc et al. 2003; Müller et al. 2004), la disponibilidad de alimento (Verboven et al. 2003; Gasparini et al. 2007), la edad de la hembra (Pilz et al. 2003; Sasváry et al. 2004) o el atractivo de la pareja (Burley 1986, 1988; Gil et al. 1999; Cunningham y Russell 2000; Tanvez et al. 2004).

En especies con reproducción sexual la competencia por parejas ha favorecido la evolución de características muy elaboradas, denominadas ornamentos o señales sexuales (Darwin 1859, 1871; Andersson 1994). Los individuos, típicamente los machos, que despliegan los ornamentos más elaborados se benefician al obtener más apareamientos o al aparearse con las hembras de mayor calidad. Mientras que las hembras que se aparean con el macho con ornamentos más elaborados o señales más intensas pueden obtener (i) beneficios directos, por ejemplo si los machos más ornamentados tienen buenos sitios para anidar, un gran territorio, invierten más en los cuidados parentales o son más saludables, con lo cual disminuye el riesgo de infecciones (Cunningham y Russell 2000; Kirkpatrick y Ryan 1991); (ii) beneficios indirectos, si los machos más ornamentados heredan "buenos genes" a su progenie, aumentando la supervivencia o el atractivo de las crías (Fisher 1930; Andersson 1994). Sin embargo, debido a que no siempre se obtiene a la pareja optima, a que la reproducción es costosa, y a que los individuos deberían de tratar de maximizar el balance en adecuación entre la inversión en la reproducción presente y futura, se esperaría que los

individuos ajustaran su inversión en la reproducción de acuerdo al beneficio esperado (Trivers 1972; Stearns 1992; Sheldon 2000).

La hipótesis de la inversión diferencial propone que las hembras apareadas con machos más atractivos deberían incrementar su inversión en la reproducción cuando los machos preferidos proveen algún tipo de beneficio directo o indirecto (Burley 1986, 1988). Esta inversión diferencial podría, además de incrementar el beneficio que se obtiene en un evento con alto valor reproductivo, aumentar las posibilidades de obtener parejas atractivas o de mantener la pareja en los subsiguientes eventos reproductivos (Burley 1986). Así, si las hembras aumentan su inversión cuando están apareadas con un macho atractivo, los efectos positivos en la adecuación de las crías serían el resultado de los efectos maternos, mas la contribución paterna directa e indirecta (Uller et al. 2005). En apoyo a esta hipótesis de inversión diferencial, se ha encontrado que en algunas especies de aves, las hembras apareadas con machos atractivos aumentan el volumen de los huevos (Cunningham y Russell 2000, Uller et al. 2005, Gilbert et al. 2006) y también modifican la composición bioquímica de estos, aumentando la concentración de andrógenos (Gil et al. 1999, 2004b, Tanvez et al. 2004, Loyeau et al 2007) o anticuerpos (Saino et al. 2002) en el vitelo de los mismos.

La inversión diferencial en relación con el atractivo del macho puede tener consecuencias evolutivas importantes en la expresión de los caracteres sexuales secundarios, modificando la tasa y la dirección evolutiva de estas señales (Kirkpatrick y Lande 1989; Sheldon 2000). Estudios experimentales han mostrado que las condiciones durante el desarrollo pueden influir en la expresión de señales (características sexuales secundarias), sobre todo cuando se trata de características dependientes de la condición, afectando así el atractivo en la etapa adulta de las crías (Gustafsson et al. 1995; Panzica et al. 2005). Tanto en roedores como en aves se ha visto que la exposición a los esteroides durante la ontogenia afecta la expresión de señales en la etapa adulta (Adkins 1979, Clark y Gales 1998, Strasser y

Schwabl 2004, Panzica et al. 2005; Eising et al. 2006). Una inversión diferencial de andrógenos en los huevos podría afectar la expresión de las señales y podría tener consecuencias sobre el éxito reproductivo futuro de estos individuos (Strasser y Schwabl 2004, Panzica et al. 2005). Por lo tanto, los efectos maternos en función del atractivo de la pareja (inversión diferencial) podrían afectar la evolución de las señales sexuales además de generar variaciones en el sistema de inversión parental.

Tamaño del huevo e intervalo de puesta

En algunas especies de aves, como en el ánade real (*Anas platyrhyncos,* Cunningham y Russell 2000), la codorniz china (*Coturnix chinensis*, Uller et al. 2005) y el pinzón zebra (*Taeniopygia guttata*, Gilbert et al. 2006) se ha demostrado experimentalmente que, de acuerdo al atractivo de su pareja, las hembras ajustan su inversión, modificando el tamaño del huevo. Las hembras apareadas con los machos que exhiben los ornamentos más desarrollados ponen huevos más grandes comparadas con las hembras apareadas con machos menos atractivos (Cunningham y Russell 2000, Uller et al. 2005, Gilbert et al. 2006). Gilbert et al. (2006) encontraron que los pollos nacidos de huevos mas grandes puestos por hembras apareadas con machos mas atractivos tuvieron una tasa de solicitud de alimento mayor que los pollos nacidos de huevos puestos por hembras apareadas con machos menos atractivos, y también que la tasa de crecimiento de los pollos que tuvieron un padre atractivo no disminuyó de acuerdo al orden de puesta como si ocurrió en los pollos que tuvieron padres menos atractivos.

Se ha sugerido que los huevos más grandes pueden contener más agua, proteínas o lípidos que pueden influir sobre la probabilidad de eclosión y la supervivencia de las crías durante los primeros días de vida (Croxall 1992; Amundsen et al. 1996; Price 1998;

Cunningham y Russell 2000, Nager et al. 2000; Dzialowski y Sotherland 2004, Krist et al. 2004, D'Alba y Torres 2007). Por ejemplo, en el emú (*Dromaius novaehollandiae)* existe una fuerte correlación entre el tamaño del huevo y el tamaño del pollo al nacer. Los pollos que nacen de los huevos más grandes son más pesados, más grandes (tarso y pico) y tienen órganos vitales (corazón, hígado, molleja) más grandes (Dzialowski y Sotherland 2004). Las diferencias de tamaño entre crías de una misma nidada son parámetros claves en la probabilidad de supervivencia de las mismas (Lack 1954, Mock y Parker 1997, Drummond et al. 2006). En general las crías más grandes tienen un mejor acceso a la fuente de alimento y mayor probabilidad de supervivencia que las crías más chicas (Drummond 2006). Por lo tanto, se ha sugerido que las hembras a través de una inversión diferencial en el tamaño del huevo de acuerdo al orden de puesta podrían incrementar o reducir las asimetrías competitivas de sus crías y así favorecer o limitar la reducción de la nidada según la calidad del ambiente al momento de poner los huevos (Slagsvold 1984, D'Alba y Torres 2007), y/o de acuerdo con el atractivo de su pareja (Velando et al. 2006).

No obstante, las diferencias en tamaño entre crías de una misma nidada no solamente resultan de las diferencias en tamaño entre huevos, también , en gran parte, obedecen a las diferencias en edad debido a la eclosión asincrónica (Lack 1954; Osorno y Drummond 1995). De acuerdo con la hipótesis de la reducción facultativa de la nidada, una eclosión asincrónica permite a los adultos ajustar el tamaño de la nidada de acuerdo con la disponibilidad de alimento durante el periodo de crianza. Este mecanismo es una manera eficiente de eliminar rápidamente las crías "extras" bajo condiciones de crianza sub-optimas (Lack 1954; Mock y Parker 1997; Drummond 2006). Cuando la disponibilidad de alimento es limitada, en general, la primera cría (debido a su mejor capacidad motriz y a su ventaja en término de tamaño) monopoliza la fuente de alimento asegurando su propia supervivencia, mientras que la segunda cría se ve privada de alimento y es eliminada. Sin embargo, si las condiciones de

crianza son favorables, toda la nidada sobrevive (Wiebe y Bortolotti 1995; Wiebe et al. 1998; Drummond 2006). Si los efectos maternos dependen del contexto, como fue sugerido por Mousseau y Fox (1998), y las hembras están anticipando las futuras condiciones de crianza que la progenie va a experimentar, entonces la modificación del tamaño del huevo y/o del intervalo de puesta por parte de la hembra podría ser una estrategia para amplificar o reducir las asimetrías competitivas entre crías hermanas que, a su vez, podría incrementar o disminuir la probabilidad de ocurrencia de la reducción de la nidada.

Sin embargo, aunque un gran número de estudios han mostrado efectos positivos del tamaño del huevo sobre la adecuación de las crías (revisiones en Williams 1994; Christians 2002), estos efectos parecen desaparecer después de una o dos semanas post eclosión (Krist et al. 2004; Amundsen et al. 1996), o las correlaciones entre el tamaño del huevo y la calidad de las crías no son tan claras (Nager et al. 2000). El tamaño del huevo no siempre se correlaciona con la probabilidad de eclosión (Clifford y Anderson 2002); con el peso de las crías durante el periodo de crianza (Krist et al. 2004, Smith *et al*. 1995; Amundsen *et al*. 1996; Reed *et al*. 1999; Styrsky *et al*. 1999; Risch y Rohwer, 2000), o con la supervivencia de las mismas (Smith *et al*. 1995; Amundsen *et al*. 1996; Styrsky *et al*. 1999). La revisión mas reciente a este respecto concluye que el tamaño del huevo puede conferir ciertas ventajas a las crías pero únicamente cuando las condiciones ambientales son pobres y durante un periodo de tiempo corto (Christians 2002). Estos resultados sugieren que un parámetro tal vez mas importante que el tamaño del huevo en si, podría ser su composición bioquímica (Nager et al. 2000; Royle et al. 2003).

Andrógenos en el huevo

Los andrógenos de origen materno, como por ejemplo la testosterona (T), la androstenediona (A4) y la dihidrotestosterona (DHT), están presentes en el vitelo de los huevos (Schwabl 1993, 1996 1997; Lipar et al. 1999; Gil 2003; Gil et al. 1999, 2004a y b; Eising y Groothuis 2003; Groothuis et al. 2005). Sin embrago el mecanismo por el cual las hembras transfieren estos esteroides a los huevos sigue siendo desconocido (Lipar et al. 1999; Gil 2003; Hackl et al. 2003; Groothuis et al. 2005; Marshall et al. 2005; Carere y Balthazar 2007; Groothuis y Schwabl 2008). Recientemente, Groothuis y Schwabl (2008) propusieron tres hipótesis a cerca de los mecanismos por los cuales las hembras podrían invertir o transferir (según si el mecanismo es activo o pasivo) andrógenos en el vitelo de los huevos. La primera hipótesis ("*Physiological Epiphenomenon Hypothesis*") se basa en los estudios en los cuales se encontraron correlaciones positivas entre las concentraciones de andrógenos en el vitelo del huevo y las concentraciones circulantes de las hembras (*Serinus canaria*, Schwabl 1996; *Carpodacus mexicanus*, Badyaev et al. 2005; *Junco hyemalis*, Jawor et al. 2007). Esta hipótesis propone un mecanismo de transferencia pasivo, según el cual las concentraciones de andrógenos en el vitelo deberían de reflejar las concentraciones de andrógenos circulantes en las hembras. Alternativamente, la segunda hipótesis ("*Flexible Distribución Hypothesis*") propone que existe una regulación de la distribución de los andrógenos entre la hembra y el vitelo de los ovocitos. En caso de una producción limitada de andrógenos, este mecanismo podría explicar las correlaciones negativas encontradas en ciertos estudios (*Passer domesticus*, Mazuc et al. 2003; *Larus fuscus*, Verboven et al. 2003; *Sialia sialis*, Navara et al. 2006) e implica la existencia de un compromiso ("trade-off") entre los niveles hormonales de la hembra y del vitelo. Finalmente, una tercera hipótesis ("*Independant Regulation Hypotesis*") se basa en la ausencia de correlación entre las concentraciones de hormonas en

las hembras y en el vitelo (*Sturnus vulgaris*, Pilz et al. 2003 and Williams et al. 2004; *Serinus canaria*, Marshall et al. 2005), y propone un sistema de regulación independiente, permitiendo la variación de niveles de andrógenos en el huevo sin interferir sobre los niveles circulantes de la hembra. Esta hipótesis se apoya principalmente en el hecho de que las células de las paredes del folículo en desarrollo sintetizan andrógenos a nivel local (Hackl et al. 2003; Sockman et al. 2001). Actualmente con la información disponible no es posible discernir entre estas tres hipótesis y aunque los mecanismos de deposición de los andrógenos maternos en el vitelo de los huevos no se conozcan con certeza, muchos estudios han encontrado efectos de estas hormonas sobre el desarrollo de los embriones y el fenotipo de las crías.

En las aves, además de la función bien conocida de los esteroides en la diferenciación sexual a nivel cerebral (Adkins 1979; revisiones en Balthazart et al. 1996; Schingler 1998; Panzica et al. 2005), se ha sugerido que los andrógenos podrían intervenir en otros mecanismos durante la etapa de desarrollo embrionario (Lipar y Ketterson 2000; Godsave et al. 2002; Jenkins y Porter 2004). En el pinzón cebra, los receptores a los andrógenos están presentes en el romboencéfalo para el día 7 de desarrollo embrionario aunque todavía ninguna producción endógena de esteroides sea posible (Godsave et al. 2002). La producción gonadal de esteroides necesita de un eje hipotálamo-hipófisis-gónadas funcional, con secreción de hormona liberadora de gonadotropina (GnRH) que no se detecta antes del día 11 del desarrollo (Godsave et al. 2002). A través de sus receptores para andrógenos localizados en el romboencéfalo y en la siringe, los andrógenos de origen materno influyen en componentes acústicos y visuales involucrados en la conducta de solicitud de alimento (Godsave et al. 2002). También en el tordo charretero (*Angelaius phoeniceus*) se ha mostrado experimentalmente una relación entre la concentración de testosterona y el peso del músculo *musculus complexus* (Lipar y Ketterson 2000). Este músculo está implicado en los

movimientos de flexión del cuello necesarios para romper el cascarón del huevo al momento de la eclosión y en los movimientos oscilatorios de la cabeza durante la solicitud de alimento (Lipar y Ketterson 2000). De acuerdo con este estudio, se encontraron relaciones positivas entre la concentración de testosterona en el vitelo y la conducta de solicitud de alimento en el canario (*Serinus canaria*), la gaviota reidora (*Larus ridibundus*) y el pinzón cebra (Schwabl 1996; Eising y Groothuis 2003; Von Engelhardt et al. 2006). Además, los pollos eclosionados de huevos con mayor concentración de T son mas activos, obtienen mas alimento (Eising y Groothuis 2003), tienen una tasa de crecimiento mayor (Eising y Groothuis 2003, Von Engelhardt et al. 2006), y son más competitivos (Eising et al. 2001), comparados con los pollos nacidos de huevos con menor concentración de T. La inyección de una mezcla de T y A4 en huevos de gaviotas reidoras acelera el desarrollo embrionario (Eising et al. 2001), e inyecciones de testosterona en los huevos del azulillo de garganta canela (*Sialia sialis*) estimulan el crecimiento embrionario (los pollos son más pesados al nacer), y la respuesta inmune celular de las crías (Navara et al. 2006).

La exposición a los andrógenos durante el desarrollo tiene consecuencias importantes en las conductas sexuales que se exhiben en la etapa de adulto. Estos efectos, hace tiempo conocidos, son el resultado de la diferenciación sexual a nivel cerebral (Adkins 1979; Panzica et al. 2005). Más recientemente se ha documentado que las concentraciones de testosterona en el vitelo pueden afectar otras conductas además de las sexuales en el adulto. En el gorrión común (*Passer domesticus*) se encontró una relación positiva entre el tamaño del ornamento (babero) y las concentraciones de T presentes en el vitelo en los machos, y en ambos sexos los pollos nacidos de huevos con mayor concentración del androgeno obtienen y defienden de manera mas eficiente una fuente de alimento (Strasser y Schwabl 2004). De la misma manera, en el canario, la concentración de T en el vitelo tiene un efecto positivo sobre el rango social del individuo después del emplumado (Schwabl 1993). Aun más, las gaviotas reidoras

eclosionadas de huevos que fueron inyectados con T desplegaron con mayor frecuencia conductas de defensa de territorio y cortejo un año después de su nacimiento que los individuos controles (Eising et al. 2006). También los individuos que recibieron una inyección de T en el huevo exhibieron un plumaje nupcial mas desarrollado que los individuos controles, haciéndoles parecerse mas a individuos adultos (Eising et al. 2006).

Sin embargo, aunque muchos estudios han encontrado efectos positivos de los andrógenos sobre parámetros relacionados con la supervivencia de las crías también se han reportado efectos negativos. Por ejemplo, niveles elevados de T en el vitelo pueden atrasar la eclosión (Sockman y Schwabl 2000; Von Egelhardt *et al.* 2006); disminuir la respuesta inmune humoral y celular de las crías (Müller *et al.* 2005; Navara *et al.* 2005); aumentar la tasa metabólica basal y el gasto de energía (Tobler et al. 2007); sesgar la proporción de sexo hacia machos y afectar negativamente el peso corporal de los pollos (Rubolini et al. 2006).

Estos resultados, que parecen contradictorios con la idea de que los andrógenos son benéficos, resaltan la importancia de considerar la existencia de un compromiso entre los efectos positivos y negativos de los esteroides sobre varios parámetros relacionados con la supervivencia de las crías. Por ejemplo, en el azulillo de garganta canela, la inyección *in ovo* de una dosis baja de T (0.3 µg) tiene efectos positivos sobre el crecimiento embrionario y el peso a la eclosión de las crías (Navara et al. 2006), pero la inyección de una dosis mas alta (3 µg) reduce el éxito de eclosión y la respuesta inmune celular de la crías (Navara et al. 2005). De la misma manera, los efectos de los andrógenos pueden depender del sexo del individuo, como fue documentado en el pinzón cebra, en el cual la elevación de la T en los huevos atrasa la eclosión y reduce la tasa de crecimiento de los machos, pero incrementa la solicitud de alimento en las hembras así como su tasa de crecimiento (Von Engelhardt et al. 2006). En contraste, en la gaviota reidora, los niveles elevados de T afectan negativamente la respuesta inmune celular de los machos, pero no la de las hembras (Muller et al. 2005). Estos resultados

muestran que los efectos de los andrógenos dependen de varios factores (como su concentración y el sexo del individuo) que es necesario tomar en cuenta en el momento de la interpretación de los resultados.

Adicionalmente, otra variable que podría influir sobre la interpretación de los resultados es el patrón de inversión de andrógenos de acuerdo al orden de puesta. Se ha reportado, que la concentración de testosterona en el vitelo aumenta de acuerdo al orden de puesta en los canarios (Schawbl 1997), la gaviota sombría (*Larus fuscus*, Royle et al. 2001), el estornino pinto (*Sturnus vulgaris*, Pilz et al. 2003), y la gaviota reidora (*Larus ridibundus*, Eising y Groothuis 2003) pero disminuye de acuerdo al orden de puesta en la garza garrapatera (*Bubulcus ibis*, Schwabl 1997), y en el pinzón cebra (Gil et al. 1999). Sin embargo, no se encontró diferencia en la golondrina (*Tachycineta bicolour,* Whittingham y Schwabl 2002*)*, en el bobo café (*Sula leucogaster*) y en el bobo de patas azules (Drummond et al. 2008). La variación en la concentración de T de acuerdo al orden de puesta fue interpretada por Schwabl (1997) como un mecanismo adaptativo por el cual las hembras podrían modificar la probabilidad de sobrevivencia de sus crías. Esta hipótesis fue denominada "*favoritismo parental*" (Schwabl 1997). El incremento de testosterona en el vitelo de acuerdo al orden de puesta podría ser una manera "económica" de reducir las diferencias entre crías de un mismo nido debido a una eclosión asincrónica incrementando la competitividad (vigor y solicitud de alimento) de la última cría como se ha visto en los canarios y las gaviotas (Schwabl 1997; Eising y Groothuis 2003). Alternativamente, un decremento en la concentración de testosterona de acuerdo al orden de puesta podría ser una manera de facilitar la reducción de la nidada como en el caso de la garza garrapatera o el pinzón cebra (Schwabl 1997; Gil et al. 1999).

Aunque la testosterona es la hormona mas estudiada, en la mayoría de los estudios publicados no se midió de manera específica la concentración de este androgeno. Sin el

empleo de técnicas adecuadas (como columnas de cromatografía) para aislar la testosterona de otros andrógenos presentes en la muestra, los resultados obtenidos pueden reflejar la concentración total de andrógenos (debido a las reacciones cruzadas de los anticuerpos con otros andrógenos) y no solamente la concentración de testosterona (Von Engelhardt y Groothuis 2005). Hasta hace muy poco se había considerado que los andrógenos presentes en mayor concentración en los huevos (testosterona, androstenediona y dihidrotestosterona) tenían los mismos efectos. Sin embargo recientemente, un estudio comparativo de Gil et al. (2007) ha resaltado la importancia de considerar cada hormona de manera independiente. Se ha visto que a diferencia de la concentración de T en el vitelo de los huevos, la concentración de androstenediona aumenta de acuerdo al peso corporal de la especie considerada, es decir, las aves más pesadas tienen concentraciones más altas de androstenediona en el vitelo que las especies más ligeras. . Se ha sugerido que el desarrollo embrionario de especies "grandes" requiere cantidades mayores de andrógenos y que la mejor manera de satisfacer esa demanda seria suplementar el vítelo con androstenediona evitando así los efectos tóxicos de la testosterona en altas concentraciones (Groothuis y Schwabl 2002). Como la androstenediona es el precursor de la testosterona, los embriones podrían convertir de manera progresiva la A4 en T gracias a la acción de la enzima responsable de esta conversión, la 17β-hidroxiesteroide deshidrogenasa.

Por otro lado, la concentración de androstenediona en el vitelo de los huevos está fuertemente asociada a un sistema de vida colonial y parecen alargar el tiempo relativo de incubación pero, simultaneamente,, parece acelerar el tiempo relativo de crianza en el nido (Gil et al. 2007). Este fenómeno podría tener efectos positivos al disminuir el riesgo de mortalidad de las crías por parasitismo, cuya prevalencia es mayor en las especies que presentan un periodo de crianza en el nido muy largo (Møller 2005; Gil et al. 2007). En el pirincho común, *Guira guira*, que presenta un sistema de anidamiento comunal (varias

hembras ponen sus huevos en el mismo nido), se encontró una fuerte asociación entre las concentraciones de androstenediona y el tamaño de puesta comunal (Carielo et al. 2006). Así mismo se sugirió que los niveles elevados de androstenediona podrían estar relacionados con la producción de fenotipos más competitivos en grupos sociales (Carielo et al. 2006; Gil et al. 2007). Adicionalmente, el trabajo de Yao y Shang (2005) sugiere que la androstenediona, en contraste con la testosterona, tiene un efecto inmunoestimulante. De acuerdo con este resultado, se encontró una relación positiva entre la concentración de androstenediona en el vitelo y la respuesta inmune celular de los pollos de golondrinas (*Hirundo rustica*, Gil et al. 2006), así como en los huevos de la gaviota tridáctila entre la androstenediona y la concentración de inmunoglobulinas (*Rissa tridactyla*, Gasparini et al. 2007). Estos resultados indican que es importante considerar de manera independiente cada hormona y sus efectos específicos.

El bobo de patas azules

El bobo de patas azules (*Sula nebouxii*), es una ave marina de larga vida (aproximadamente 20 años) que anida en colonias. Durante el cortejo, que puede durar varias semanas, los machos realizan una serie de despliegues ritualizados en los que exhiben de forma muy exagerada sus patas que son de color azul turquesa muy brillante (Nelson 1978; Osorio-Beristain y Drummond 1998). Recientemente se demostró experimentalmente que este color es una característica bajo selección sexual (Torres y Velando 2003 y 2005), y cuando el color de las patas del macho se modificó experimentalmente a un azul más oscuro, las hembras disminuyeron el cortejo y copularon con menos frecuencia, comparadas con las hembras en el grupo control (Torres y Velando 2003). La coloración de las patas del macho es una característica muy dinámica (puede cambiar en periodos de 24 horas), y está relacionada con

el estado nutricional y de salud del individuo (Velando et al. 2006). Los machos en un buen estado nutricional exhiben un color de patas azul turquesa a diferencia de los machos en condición nutricional pobre que tienen un color de patas azul oscuro (Velando et al. 2006). Esta señal depende en parte de los carotenoides obtenidos de la dieta, los machos suplementados con carotenoides exhibieron un color mas turquesa y brillante que los machos controles y también tuvieron una mejor respuesta inmune celular que los últimos (Velando et al. 2006).

Se ha indicado que la información expresada en los tegumentos probablemente difiere de la información reflejada en la coloración del plumaje (Hill 1990, Lozano 1994). La coloración del plumaje que los individuos despliegan durante el cortejo resulta de procesos que pueden llevar semanas o meses en los cuales se producen las plumas y se van acumulando pigmentos en ellas, por lo anterior se ha sugerido que la coloración del plumaje podría reflejar la condición general y disponibilidad de recursos que el individuo tenía al momento de la muda pero no necesariamente indica su condición actual (Lozano 1994). En contraste, el color de los tegumentos puede reflejar la condición fisiológica más reciente porque varía rápidamente en función de las condiciones ambientales por lo cual puede ser un buen indicador de la aptitud de forrajeo del individuo (Hill 1990, Lozano 1994, Pérez-Rodríguez y Viñuela 2008).

En el bobo de patas azules el macho y la hembra participan en la incubación y el cuidado de las crías (cuidado bi-parental; Drummond et al. 1986; Guerra y Drummond 1995). El tamaño de puesta modal es de dos huevos y el cuidado parental es muy largo: 42 días de incubación y alrededor de 4 a 5 meses de crianza (Drummond et al. 1986; Torres y Drummond 1999). La contribución del macho durante la crianza es considerable y afecta el éxito reproductivo de las hembras (Guerra y Drummond 1995; Velando y Alonso-Alvarez 2003). Una disminución experimental de la aportación del macho durante esta etapa afecta

negativamente la condición corporal de la hembra, y probablemente sus futuros eventos reproductivos (Velando y Alonso-Alvarez 2003).

Se ha sugerido que el color de las patas en el bobo de patas azules podría ser un indicador del esfuerzo paterno, permitiendo a las hembras evaluar a la pareja y ajustar su inversión en la puesta de acuerdo a la condición del macho (Velando et al. 2006). En un experimento en el cual las crías fueron intercambiadas entre nidos, el color de la patas del macho adoptivo fue la variable que explico la mayor parte de la varianza observada en la condición corporal de las crías (Velando et al. 2005). Aún más, las hembras ajustaron su inversión en la puesta en función del color de las patas de su pareja, disminuyendo el volumen del huevo cuando el color de las patas del macho se modificó a un azul más oscuro (Velando et al. 2006) un parámetro relacionado positivamente con la probabilidad de eclosión en esta especie (D'Alba y Torres 2007).

Los pollos de esta especie nacen de manera asincrónica (3.9 ± 1.5 días de diferencia, D'Alba y Torres 2007), compiten agresivamente (picotazos y mordidas) para obtener el alimento que traen los padres, y esta competencia, en caso de escasez de alimento, lleva con frecuencia a la muerte de alguna de las crías, típicamente la de menor edad (Drummond et al. 1991; Osorno y Drummond 1995). A lo largo de los 15-20 primeros días de crianza se establece una jerarquía donde generalmente el segundo pollo de una nidada de dos adopta un papel de subordinado, respondiendo a la conducta agresiva de su hermano mayor con una postura de sumisión ritualizada (Drummond y Osorno 1992). Los pollos subordinados tienen niveles de corticosterona en la sangre mas elevado que los dominantes o los pollos únicos (Nuñez de la Mora 1996), y la administración experimental de corticosterona en los pollos subordinados parece favorecer de manera espontánea la postura de sumisión (Vallarino et al. 2006). La hipótesis de "*behavioural feedback*" (Leshner 1975, 1980) propone que los cambios hormonales que ocurren durante las interacciones agresivas afectan la conducta actual del

individuo así como su conducta futura en situaciones similares. Por lo tanto la corticosterona podría favorecer la agresión en pollos ganadores de pelea y al mismo tiempo favorecer la expresión de conducta de sumisión en los individuos que perdieron la "pelea". De acuerdo con esta hipótesis se encontró que, en los pollos dominantes, la corticosterona parece estar involucrada en el control de la agresión, mientras que no se encontró ninguna relación entre la testosterona en plasma y la agresión de crías en sus dos primeras semanas de vida (Ramos Fernandez et al. 2000). No obstante, la falta de relación entre la testosterona y la agresión requiere mas investigación dado que en 48% de los casos no se pudo detectar la hormona (Ramos-Fernández et al. 2000). Recientemente, Drummond et al. (2008) rechazaron para el bobo de patas azules la hipótesis del favoritismo parental propuesta por Schawbl (1997) que establece que una disminución en la cantidad de andrógenos en los huevos de acuerdo al orden de puesta podría favorecer la reducción de la nidada. No se encontró diferencia en la concentración de testosterona y dihidrotestosterona entre el primer y segundo huevo, aunque se encontró una tendencia negativa marginalmente significativa para la androstenediona (Drummond et al. 2008).

Sin embargo, si los efectos maternos son dependientes del contexto (Mousseau y Fox 1998), la inversión de la hembra en términos de tamaño del huevo o de la concentración de andrógenos en el vitelo podría variar en función de las condiciones ambientales y el atractivo de la pareja. Por lo tanto, una inversión mayor (o menor) de recursos en los huevos de acuerdo al ambiente y al atractivo del macho podría representar una ventaja (o desventaja) muy grande para un pollo permitiéndole ser un mejor (o peor) competidor y así favorecer (o limitar) la reducción de la nidada (Schwabl 1993, 1997).

Esquema general de la tesis

En esta tesis evaluamos el papel del color de las patas de los machos en la inversión en la puesta por parte de las hembras del bobo de patas azules. En el primer estudio pusimos a prueba la hipótesis de que las hembras ajustan su inversión dependiendo de las condiciones esperadas durante la crianza ("*context dependant allocation*", Mouseaux y Fox 1998). En particular evaluamos si el color de las patas de los machos durante la puesta, una señal honesta de la condición del macho, afecta la inversión en los huevos por parte de las hembras (hipótesis de inversión diferencial, Burley 1986, 1988). Para evaluar la hipótesis anterior, durante dos años consecutivos con condiciones ambientales muy distintas (un año de El Niño y un año con buenas condiciones para la crianza), un día después de la puesta del primer huevo manipulamos el color de las patas del macho a un azul más oscuro, parecido al color de un macho en baja condición, y medimos la inversión de la hembra en el segundo huevo. La inversión en el segundo huevo se estimó como el volumen y masa del huevo, el intervalo de puesta, y la concentración de andrógenos (androstenediona, testosterona y dihidrotestosterona) en el vitelo. Si las hembras ajustan su inversión en la puesta en función de las condiciones esperadas de crianza, esperamos que en un año con condiciones ambientales pobres, para mitigar las asimetrías entre hermanos, las hembras aumenten su inversión en el segundo huevo con respecto al primer huevo comparado con un año con buenas condiciones ambientales. Sin embargo, si el color de las patas del macho se ve deteriorado indicando un decremento en su condición y posiblemente en su esfuerzo paterno, esperamos que la inversión de la hembra en el segundo huevo disminuya comparada con los segundos huevos de nidos en el cual el color del macho no se modifico, especialmente en años con condiciones ambientales pobres. Este articulo esta publicado en la revista *Behavioral Ecology and Sociobiology*, (Dentressangle et al. 2008).

En el segundo capitulo de esta tesis, con el objetivo de evaluar la hipótesis de que las hembras favorecen la reducción de la nidada a través de su inversión diferencial en los huevos cuando están apareadas con machos con un color de patas menos atractivas, realizamos la misma manipulación del color de las patas en machos durante la puesta y registramos el éxito de eclosión de los huevos, la concentración plasmática de andrógenos en los pollos a los 7 días de edad y su respuesta inmune celular a los 15 días de edad. Si las hembras favorecen la reducción de la nidada cuando la condición de su pareja (reflejada en el color de las patas) se ve deteriorada esperamos que el éxito de eclosión de los segundos huevos sea menor en el grupo de machos en el cual se disminuyó experimentalmente el atractivo del color de las patas comparado con el grupo control. Si la concentración de andrógenos en el vitelo se correlaciona con la de andrógenos en el plasma de las crías a los 7 días de edad (Gil 2003), esperamos que las segundas crías que provienen de un nido en el cual se simuló un deterioro en la condición del macho tengan menor concentración de andrógenos a los 7 días de edad comparado con las crías de los nidos controles. Además, si la androstenediona en el vitelo estimula la respuesta inmune de las crías (Gil et al. 2006), esperamos que las segundas crías del grupo experimental tengan una respuesta inmune celular menor a las crías del grupo control. Este articulo esta en revisión en la revista *Behavioral Ecology*.

Finalmente, para evaluar los efectos de la inversión diferencial en los huevos sobre la conducta de las crías relacionada con sus habilidades competitivas y su supervivencia, manipulamos el color de las patas de los machos un día después de la puesta del primer huevo para simular un deterioro en su condición y cuando las segundas crías de estos nidos cumplieron 10 días de edad registramos su conducta agonística y de alimentación. Las crías se pesaron al nacer así como a los días 7 y 15 para determinar la tasa de crecimiento. A los 7 días de edad, se tomo una muestra de sangre para medir las concentraciones de andrógenos en el plasma y a los 10 días de la segunda cría, registramos las conductas de agresión (picotazos y

mordidas) y de solicitud de alimento en nidadas experimentales y controles de dos crías. Si los andrógenos tienen un efecto positivo sobre el crecimiento, la conducta de solicitud de alimento y de agresión esperamos que las segundas crías en las nidadas experimentales tengan una tasa de crecimiento mas lenta y que soliciten y agredan con menos intensidad que las segundas crías de los nidos controles. Este articulo esta en preparación para enviar a la revista *Animal Behaviour*.

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Behav Ecol Sociobiol
DOI 10.1007/s00265-008-0620-6

ORIGINAL PAPER

Maternal investment in eggs is affected by male feet colour and breeding conditions in the blue-footed booby, *Sula nebouxii*

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Received: 21 September 2007 / Revised: 14 May 2008 / Accepted: 5 June 2008

Abstract Females are expected to vary investment in offspring according to variables that may influence the offspring fitness in a way that optimises her inclusive fitness for a particular context. Thus, when sexual ornaments signal the quality of the male, females might invest in reproduction as a function of the attractiveness of their mate. We tested whether breeding conditions and male feet colour influence reproductive decisions of blue-footed booby females. In the blue-footed booby, male feet colour is a dynamic condition-dependent sexually selected trait that is related to paternal effort. During two consecutive years, an El Niño year (poor breeding conditions) and a year with good breeding conditions, we experimentally reduced male attractiveness by modifying their feet colour after the first egg was laid and recorded female investment in the second egg. We found that, relative to the first egg in the clutch, females laid heavier second eggs during the poor year than during the good year. Females paired with males with duller feet colour reduced second-egg mass and volume and delayed the laying of the second egg, independently of the year. Absolute yolk androstenedione (A4) concentration (but not testosterone, T) in second eggs was higher during a poor year than during a good year. Only during a year with poor breeding conditions, females paired with experimental males decreased the relative A4 concentration (but not T) in the second egg compared to control females. Thus, blue-footed booby females probably favour brood reduction by decreasing egg quality and increasing size asymmetry between chicks when the breeding and the mate conditions are poor.

Keywords Sexual traits · Egg quality · Laying asynchrony · Yolk androgens · *Sula nebouxii* · Maternal effects

Communicated by J. Graves

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Introduction

Females are expected to vary investment in offspring according to variables that may influence the offspring fitness in a way that optimises her inclusive fitness for a particular context (Mousseau and Fox 1998a, b; Christians 2002; Verboven et al. 2003; Sockman et al. 2006; Sheldon 2000). Accordingly, in some bird species, females allocate resources in relation to variables that may influence both the offspring and the mother fitness, such as environmental conditions (Mousseau and Fox 1998a, b; Gil 2003, Gil et al. 2004a), breeding density (Mazuc et al. 2003; Müller et al. 2004) and chick sex (Müller et al. 2003; Saino et al. 2003). Furthermore, when sexual ornaments signal the quality of the male, females might adjust the level of their investment in reproduction as a function of the attractiveness of their mate (differential allocation hypothesis, Burley

1986, 1988). Accordingly, in some bird species, females paired with attractive males modify investment in eggs producing larger eggs (Cunningham and Russell 2000; Uller et al. 2005) or vary the levels of yolk testosterone (Gil et al. 1999, 2004b; Tanvez et al. 2004), antibodies (Saino et al. 2002a) and antioxidants (Saino et al. 2002b; Williamson et al. 2006).

The amount and quality of resources allocated to eggs by mothers often has a strong influence on the behaviour, growth and survival of the progeny (Schwabl 1993; Williams 1994; Christians 2002; Groothuis et al. 2005). In birds, egg size is positively correlated with hatching probability, and early growth and survival of the young (Williams 1994; Christians 2002). In addition, increasing evidence indicates that variations in maternally derived yolk androgens during early development can have positive effects on chick development (Schwabl 1996; Eising et al. 2001; Eising and Groothuis 2003; Navara et al. 2006), though negative effects have also been reported (Sockman and Schwabl 2000; Müller et al. 2005; Navara et al. 2005; Rubolini et al. 2006; Von Engelhardt et al. 2006; Tobler et al. 2007). For instance, high yolk testosterone concentrations have been shown to enhance begging behaviour and thereby the amount of food received by black-headed gull chicks (*Larus ridibundus*; Eising and Groothuis 2003) and the social rank after fledging of canary chicks (*Serinus canaria*, Schwabl 1993). Furthermore, in asynchronously hatching clutches, increased androgens in the last egg could compensate for competitive asymmetries among brood mates (Schwabl 1993, 1997; Sockman and Schwabl 2000; Eising et al. 2001; Sockman et al. 2006; Sandell et al. 2007).

Besides modifying egg quality, females may influence offspring fitness by varying the laying interval between eggs and consequently the degree of asynchrony at hatching, a key trait for sibling competition (Lack 1954; Mock and Parker 1997; Drummond 2006). Hatching asynchrony determines initial differences in size and development among brood mates which can be amplified by sibling competition affecting the nutritional, physiological and social asymmetries and survival between chicks (Mock and Parker 1997; Drummond 2006). Furthermore, parental adjustment of the degree of hatching asynchrony to the abundance or predictability of food has been interpreted as an adaptation to optimise the number and quality of offspring under variable breeding conditions (Lack 1954; Wiebe and Bortolotti 1995; Wiebe et al. 1998). Thus, by modifying the laying interval and consequently the degree of hatching asynchrony, females may facilitate brood reduction.

In the blue-footed booby, male feet colour is a dynamic condition-dependent sexually selected trait (Torres and Velando 2003; Velando et al. 2006). Preferred males, those with bright green–blue feet, have better nutritional and health condition compared to males with duller blue feet, and feet coloration can change in less than 48 h if no food is provided (Torres and Velando 2003; Velando et al. 2006). A cross-fostering experiment showed that blue-footed booby offspring condition correlates with the feet colour of the foster father, and to a lesser degree, with the feet colour of the genetic father (Velando et al. 2005). Furthermore, females decreased second-egg volume when the male feet colour was manipulated to a duller blue after the first egg was laid (Velando et al. 2006), and smaller eggs have lower hatching success and produce lighter chicks at hatching (D'Alba and Torres 2007). In this experiment, we further investigated how females adjust their investment in eggs after a sudden deterioration of male feet colour. Less than 24 h after the first egg was laid, feet colour of experimental males was modified to duller blue, mimicking males in low condition, and female investment in second egg's mass, volume and yolk androgen concentration and laying interval was measured. Reproductive success of the blue-footed booby is strongly influenced by climatic variation related to El Niño Southern Oscillation, which may affect the onset of breeding, the growth of chicks and the rate of nest abandonment (Drummond et al. 1986; Wingfield et al. 1999). To evaluate the effects of annual environmental variability on maternal investment in eggs, the experiment was repeated during two consecutive years with contrasting environmental breeding conditions: 2005, an El Niño year with poor breeding conditions (mean reproductive success of the colony 0.18 fledglings produced per total nests) and 2006, a year with good breeding conditions (1.43 fledglings produced per total nests; Drummond H. and Torres R., unpublished data). If females adjust investment within the clutch according to expected breeding conditions, we predicted that, to mitigate the asymmetries between brood mates, during a poor year females should increase investment in second eggs (egg volume, mass and yolk androgen concentration) compared to investment in second eggs during a good year. Yet, if the mate feet colour deteriorates indicating a decrease in male condition, compared to females in the control group, females paired with males with duller feet colour should decrease second-egg mass, volume and yolk androgen concentration and may delay the laying of the second egg, particularly in years with poor breeding conditions.

Materials and methods

The study was carried out in the breeding colony of blue-footed boobies at Isla Isabel, off the Pacific cost of Mexico (25° 52′ N, 105° 54′ W), from March to April 2005 and from January to May 2006.

The blue-footed booby

The blue-footed booby is a long-lived bird with a modal clutch size of two eggs (Drummond et al. 1986), laid with an average interval of 3.9±1.5 days (D'Alba and Torres 2007); and the laying interval is positively related to the hatching interval between chicks (D'Alba and Torres 2007). Both parents incubate the clutch for roughly 41 days and care for the characteristic brood of two chicks during approximately 4 months (Drummond et al. 1986; Torres and Drummond 1999). Chicks hatch on average at an interval of 4 days and the resulting asymmetry in size and development between them facilitates the establishment of a dominant–subordinate relationship (Drummond et al. 1991; Osorno and Drummond 1995). Typically, the senior outcompete the younger chick, resulting in differential mortality of the subordinate nestling (in 136 two-chick broods, 20.56% of junior chicks died while 5.88% of senior chicks died, Drummond et al. 1986). Male contribution to parental effort seems to be important for female breeding success: experimental reduction of paternal effort had a negative effect on the condition and probably future reproduction of females (Velando and Alonso-Alvarez 2003).

Experimental modification of male feet colour

Courting pairs were monitored daily to determine laying date. We manipulated feet colour of 26 males during a poor year (2005) and 64 males during a good year (2006) less than 24 h after the first egg was laid. Males were captured, banded with a numbered metal ring and randomly assigned to either the experimental or the control group. In the experimental group, feet colour was modified to a duller blue with a non-toxic intensive makeup to mimic a male in low condition (Torres and Velando 2003). In the control group, we simulated the manipulation (using a crayon in a plastic bag) without changing the original male feet colour (Torres and Velando 2003). This method of colour modification has been used before with no effects on bird behaviour and the artificial colour on experimental males lasts for 5–6 days (Torres and Velando 2003). Total handling time per bird was less than 5 min. Feet colour was measured with a spectrophotometer (MINOLTA 2600d) before and after the manipulation. Before the manipulation, feet colour of control and experimental males did not differ (peak of maximum reflectance: control 514.8±2.80 nm, experimental 518.4±2.98 nm; Mann–Whitney test, N=48, U=214.5, P=0.09; total reflectance: control 1,296.90±20.93, experimental 1,335.26±34.56; $t_{1,\ 46}$=0.93, P=0.35). Also, previous to manipulation, feet colour of males during a poor and a good year did not differ (peak of maximum reflectance Mann–Whitney test, N=48, U=205.5, P=0.10; total reflectance $t_{1,\ 46}$=2.14, P=0.38). After the manipulation, the peak of maximum reflectance on the feet colour of experimental males decreased 11.18% (460.4±0.40 nm), and total reflectance decreased 46.4% (715.11±39.99); nevertheless, the lower values remained within the natural range of variation reported for courting males in the same population (Velando et al. 2006).

Nest monitoring and yolk sampling

Nests were checked daily until the complete clutch had been laid, and all freshly laid eggs were marked with a non-toxic pen. Egg mass was determined with an electronic balance (±0.1 g), and their maximum length and width were measured with a calliper (±0.1 mm) to calculate egg volume in cubic centimeter (length × width^2 × 0.51/1,000; Hoyt 1979). Less than 24 h after laying, a yolk sample (15–20 mg) was obtained by introducing a syringe (needle type 21G) into the egg through a small hole in the shell (Schwabl 1993). The hole was sealed using a tiny drop of dentistal cement (VIARDEN, Mexico) and immediately after the egg was placed back in the nest. Yolk samples were stored in liquid nitrogen until laboratory analyses were performed. Overall, hatching success for manipulated eggs was low: 21.6% of the eggs that were yolk-sampled hatched. For the analysis, clutches of two and three eggs were used: 12 two-egg clutches (five from the experimental group and seven from the control group) from 2005 and 26 two-egg clutches (13 in the experimental group and 13 in the control group) and 14 three-egg clutches (five in the experimental group and nine in the control group) from 2006. In 2006, clutch size did not differ between treatments (Mann–Whitney test, N=52, U=964.5, P=0.63), and the number of eggs in a clutch did not have an effect on the second-egg volume (general linear model (GLM), $F_{2,\ 50}$=0.39, P=0.67), mass (GLM, $F_{2,\ 50}$=0.17, P=0.86) or androgen concentration (GLM, A4, $F_{2,\ 48}$=0.40, P=0.52; T, $F_{2,\ 49}$=0.08, P=0.77). Hence, the first two eggs from these three-egg clutches were included in the analyses. Females that failed to lay a second egg were not included in the analysis (14 in 2005 and 18 in 2006).

Hormone assays

Yolk androgen concentrations were determined by radio immune assay (RIA, Schwabl 1993). In order to extract androgens, 10–15 mg of yolk were homogenised in 1 ml of distilled water; 0.5 ml of this homogenised solution was mixed with 5 ml of diethyl ether and vortexed during 1 min. The ether phase was decanted after snap freezing in an alcohol bath at −30°C and evaporated. The dried extract was redissolved in 1 ml of isooctane. This wet extract passed through celite chromatography columns under

nitrogen flux in order to separate androstenedione (A4), dihydrotestosterone (DHT), and finally testosterone (T), using 3.5 ml of isooctane (100%), 3.5 ml of isooctane and ethyl acetate (95%:5%) and 3.5 ml of isooctane and ethyl acetate (85%:15%), respectively. Each extract was evaporated and redissolved in 1 ml of phosphate buffer (0.1 M with 1% of gelatin). The rest of the method followed the standard RIA technique (Wingfield and Farner 1975; Wingfield et al. 1999).

All androgens were determined in duplicate and were incubated overnight at 4°C with 5,000 cpm with its respective [H^3] androgen before the quantification. Duplicate values of each sample were compared to a standard curve that ranged in concentration from 12.5 to 400 pg for A4, from 9.9 to 316.8 pg for T and from 6.25 to 200 pg for DHT. The mean recovery values were 64% for A4, 57% for DHT and 58% for T. The coefficient of variation inter-assay was 6.87% for A4 and 8.38% for T, while intra-assay variation was 3.07% for A4 and 3.24% for T. Specific A4 and DHT antibodies were provided by MP Biomedicals, LLC, OH, USA (catalogue number; A4: 61320 and DHT: 61340). T antibodies were provided by World Health Organisation RIA Reagent Programme (catalogue number: K200710). The cross-reactivities of the antibodies were A4: T=4.5%, T:A4=3.5%, T:DHT=1.3%, DHT:A4=2.4% and DHT:T=22.7%.

Statistical analysis

The mass, volume and absolute concentration of yolk androgens of second eggs were analysed using general mixed models in PROC MIXED in SAS with normal error distribution (SAS Institute 1999) and the Satterthwaite approximation for the denominator degrees of freedom (Littell et al. 1996). The models included clutch identity as a random factor and the treatment, year and either mass, volume or yolk androgens of first eggs as fixed factors. As variation in yolk androgen concentrations between females is big and may have an intrinsic component that determine to some extent hormones deposition (Sandell et al. 2007; Tobler et al. 2007), yolk androgen concentrations of the first egg were included in the models. Because our experimental manipulation was performed after the first egg in the clutch was laid, and egg allocation adjustment within the clutch is probably relevant in the blue-footed booby, we also analysed whether females adjust androgens transferred to the second egg relative to the first egg in the clutch. Relative concentrations were calculated as (A4 or T concentration in the second egg×100 / A4 or T in the first egg)−100. Positive values indicate that second eggs received more A4 or T than the first egg and negative values indicate the contrary. After a significant interaction post hoc comparisons were performed using *t* test. Laying date was initially included in all models but was not significant ($P>0.05$ in all cases); we therefore excluded this variable from analysis. Concentrations of yolk DHT could not be detected in both eggs in 29 out of the 52 clutches; then, this androgen was not analysed. Laying intervals of control and experimental clutches were compared with a generalised linear model with Poisson error distribution. Mean ± SE are shown throughout the manuscript and $P<0.05$ was considered significant.

Results

First-laid eggs (that is, eggs laid before the experimental manipulation) from control and experimental pairs did not differ in mass (treatment, $F_{1, 48}=1.15$, $P=0.28$; treatment × year $F_{1, 48}=0.23$, $P=0.63$), volume (treatment, $F_{1, 48}=0.70$, $P=0.41$; treatment × year $F_{1, 48}=0.44$, $P=0.10$) and concentration of T (treatment, $F_{1, 47}=0.44$, $P=0.51$; treatment × year $F_{1, 47}=3.25$, $P=0.08$; Table 1); although during the good year, first eggs in the control group had greater concentrations of A4 than fist eggs in the experimental group (treatment, $F_{1, 46}=0.01$, $P=0.94$; treatment × year $F_{1, 46}=7.93$, $P=0.007$, Table 1, post hoc: $t_{1, 39}=2.48$, $P=0.01$). When comparing between years, first eggs in a good year were heavier (61.32±0.67 g in 2006 and 56.91±1.37 g in 2005; $F_{1, 48}=6.82$, $P=0.01$) and had more T (20.52±1.65 pg/mg of yolk in 2006 and 14.08±1.68 pg/mg of yolk in 2005; $F_{1, 49}=4.26$, $P=0.044$) than first eggs in the El Niño year of 2005, but they did not differ in size (56.83±0.74 cm^3 in 2006 and 54.04±1.12 cm^3

Table 1 Mean ± SE concentrations of yolk androstenedione (A4) and testosterone (in picogram per milligram of yolk) of first and second eggs from clutches in the control and experimental groups during a year with poor breeding conditions (2005) and a year with good breeding conditions (2006)

Androgens	Poor year (2005)		Good year (2006)	
	Control (*N*=7)	Experimental (*N*=5)	Control (*N*=22)	Experimental (*N*=18)
A4				
First eggs	307.43±74.15	586.31±134.45	704.31±83.05	466.11±36.78
Second eggs	541.55±138.35	645.45±139.99	586.83±53.74	503.40±35.75
Testosterone				
First eggs	10.75±1.07	18.74±2.67	22.39±2.33	18.36±2.27
Second eggs	16.68±1.80	19.74±2.21	17.48±2.28	18.31±1.50

in 2005; $F_{1, 48}$=3.61, P=0.06) nor in yolk concentrations of A4 (602.01±53.06 pg/mg of yolk in 2006 and 423.64± 78.78 pg/mg of yolk in 2005; $F_{1, 48}$=3.45, P=0.069).

Effects of male feet colour modification on egg investment

Egg mass and volume

As predicted, females paired with males with duller feet laid lighter second eggs than females in the control group (Table 2; Fig. 1a). The mass of second eggs differed between years and was positively related to the mass of the first egg in the clutch (Table 2), but the interaction between treatment and year was not significant (P=0.92). Second eggs were slightly heavier in the poor year than in the good year, when the mass of the first egg was controlled for (60.11±0.89 g in 2005 and 59.35±0.40 g in 2006; Table 2). The experimental manipulation of male feet colour had a similar effect on egg volume. Females in the experimental group laid smaller second eggs than control females (Table 2; Fig. 1b). The volume of second eggs was positively related to the volume of the first egg in the clutch, but there were no differences in the volume of second eggs between years (Table 2). After controlling for the volume of the first egg in the clutch, the volume of second eggs in the experimental group was 2.97% smaller than in the control group (Table 2).

Laying asynchrony

Females paired with experimental males deferred laying of the second egg in the clutch. Irrespective of year, experimental females delayed on average 0.77 days more than control females the laying of the second egg (treatment $F_{1, 48}$=6.37, P=0.01; year $F_{1, 48}$=0.14, P=0.70; treatment × year $F_{1, 48}$=0.93, P=0.33; Fig. 2).

Table 2 Comparison of mass and volume of second eggs laid by 23 experimental females and 29 control females

Model and terms	*df*	*F*	*P*
Second-egg mass			
Treatment	1, 47	7.02	0.01
Year	1, 47	11.48	0.001
First-egg mass	1, 47	46.76	<0.0001
First-egg mass × year	1, 47	10.26	0.002
Second-egg volume			
Treatment	1, 47	28.33	<0.0001
Year	1, 47	1.73	0.19
First-egg volume	1, 44.5	184.73	<0.0001
First-egg volume × treatment	1, 47	25.91	<0.0001

In the experimental group, less than 24 h after the first egg was laid, male feet colour was manipulated to a duller blue. Data were analysed with general linear mixed models. The models included the identity of the nest as a random factor (Wald Z tests for random effects: egg mass, Z=4.22, P<0.001; egg volume, Z=4.85, P<0.001). The initial models included all second-degree interactions but non-significant terms were excluded to obtain the minimum adequate model

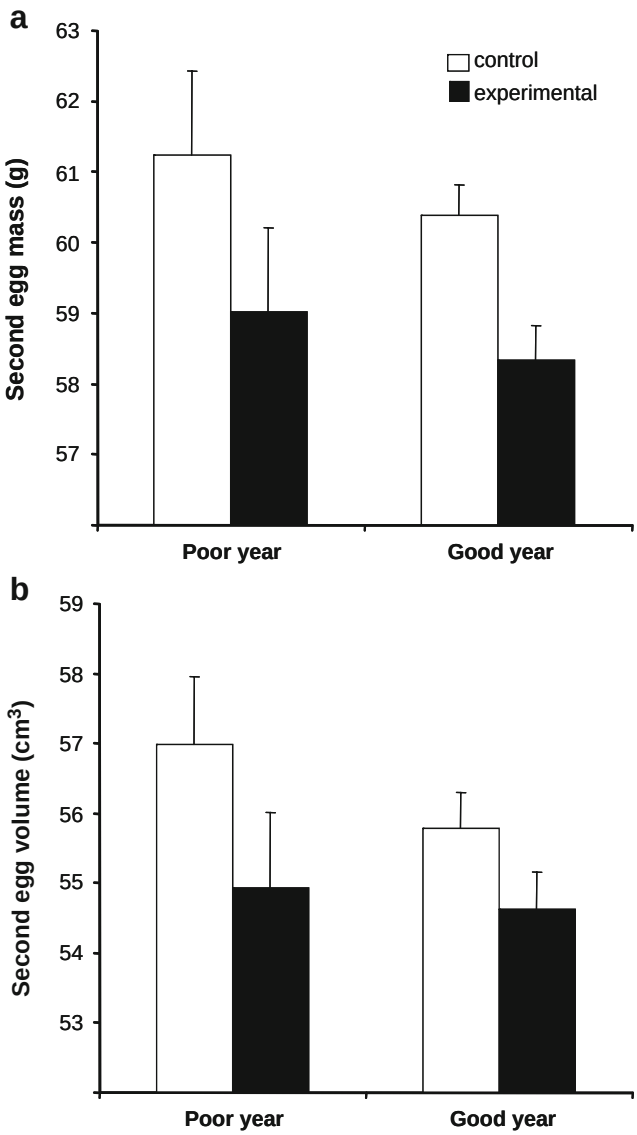

Fig. 1 Least squares means (LSM ± SE) of second-egg (**a**) mass and (**b**) volume from control and experimental clutches during a year with poor breeding conditions (control, N=7; experimental, N=5) and during a year with good breeding conditions (control, N=22; experimental, N=18). LSM were estimated from the models that included first-egg mass or volume, treatment and year of study as fixed factors and nest id as a random factor

Absolute yolk androgen concentrations

The concentration of A4 in second eggs did not differ between control and experimental groups ($F_{1, 44}$=2.30, P=0.13; all interactions with treatment P>0.12, Table 1). Concentration of A4 of first and second eggs within the clutch was positively correlated and variation among

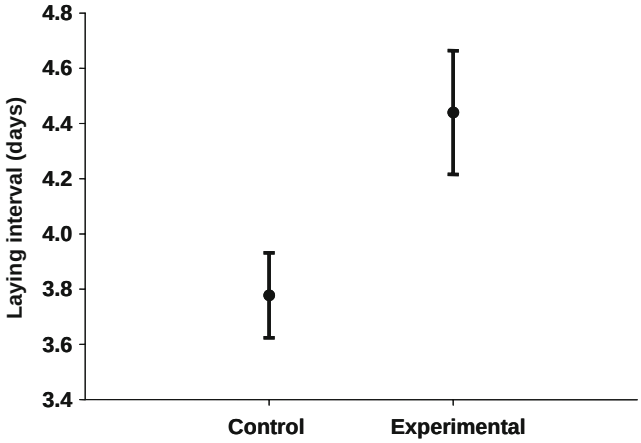

Fig. 2 Laying interval (days) between first and second eggs in the control and experimental groups from the pooled sample of both years of study

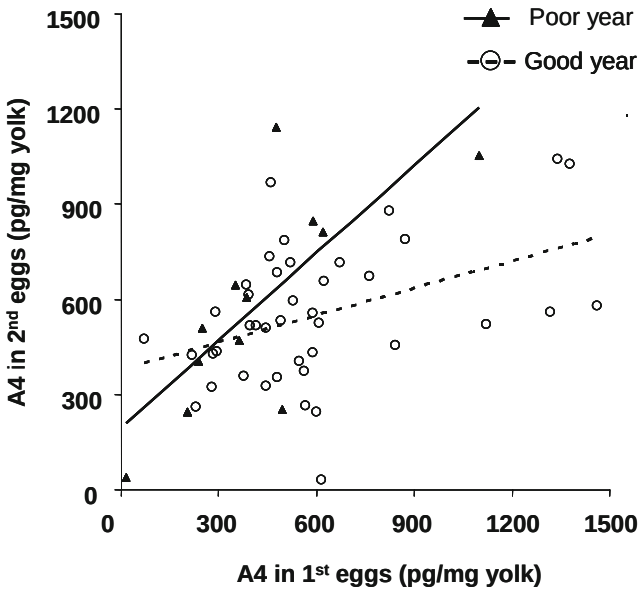

Fig. 3 Yolk A4 concentration of first and second eggs during a year with poor breeding conditions (2005) and a year with good breeding conditions (2006)

clutches was significant (first egg A4, $F_{1,\ 44}$=28.84, P< 0.0001; random effect Z=4.69, P<0.001). Yet, A4 concentration in second eggs was on average 6.69% greater in the poor year (584.85±96.42 pg/mg of yolk) than in the good year (548.17±33.51 pg/mg of yolk), when variation in the concentration of A4 of the first egg was controlled (year $F_{1,\ 44}$=3.46, P=0.069, year × first egg A4, $F_{1,\ 44}$=9.00, P=0.004, Fig. 3).

The concentration of T in second eggs did not differ between experimental treatments ($F_{1,\ 42}$=0.30, P=0.58; all interactions with treatment P>0.51) or years ($F_{1,\ 42}$=0.14, P=0.71, all interactions P>0.51). There was significant variation among clutches in the concentration of T (random effect Z=4.52, P<0.001), but the concentration of T in the first and the second egg was not related ($F_{1,\ 42}$=0.60, P=0.44).

Relative allocation of androgens

The relative allocation of A4 differed between years and the interaction between treatment and year was significant (Table 3; Fig. 4). Overall, second eggs received on average relatively more A4 than first eggs during a poor year than during a good year, and post hoc comparisons showed that, compared to the control group, females paired with experimental males decreased their relative allocation of A4 in second eggs during a poor year (2005, $t_{1,\ 10}$=6.81, P=0.01; Fig. 4) but not in a good year (2006, $t_{1,\ 38}$=2.14, P=0.15, Fig. 4).

Second eggs received relatively more T than first eggs during a poor year than during a good year (Table 3). There was a significant interaction between the treatment and the year, but this was because the control groups differed between years ($t_{1,\ 27}$=11.53, P=0.001). During a good year the relative concentration of T in control and experimental clutches did not differ (2006, $t_{1,\ 39}$=2.45, P=0.12), yet, during a poor year, experimental clutches had lower relative T concentration than controls, although this difference did not reach significance (2005, $t_{1,\ 10}$=3.47, P=0.068).

Discussion

This study suggests that maternal investment in eggs in the blue-footed booby is influenced by expected conditions during chick development (Mousseau and Fox 1998a, b; Verboven et al. 2003; Sandell et al. 2007). Females were

Table 3 Relative concentration of androstenedione (A4) and testosterone (T) in second eggs

Terms in model	*df*	*F*	*P*
A4 in second eggs			
Treatment	1, 46	2.46	0.12
Year	1, 46	9.20	0.004
Treatment × year	1, 46	8.95	0.004
T in second eggs			
Treatment	1, 47	0.77	0.38
Year	1, 47	4.08	0.049
Treatment × year	1, 47	5.70	0.02

Data were analysed with general linear mixed models. The models included the identity of the nest as a random factor (Wald Z tests for random effects: A4, Z=3.64, P=0.0001; T, Z=4.85, P<0.001). Sample size were 22 experimental and 28 control clutches for the analysis of A4 and 22 experimental and 29 control clutches for the analysis of T. The initial models included egg volume and egg mass

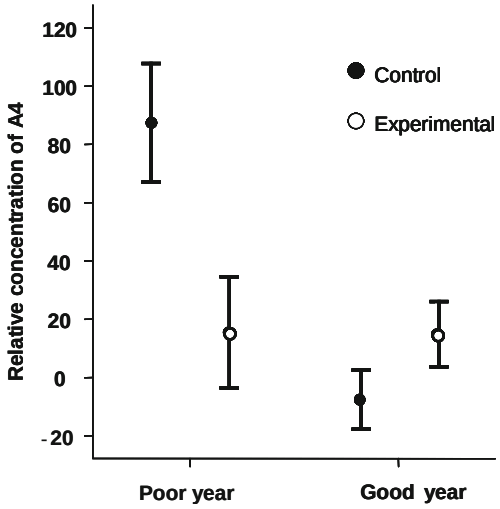

Fig. 4 Relative concentration of A4 in second eggs of control and experimental clutches during a year with poor breeding conditions (2005) and a year with good breeding conditions (2006). Relative concentration=([A4 in the second egg]×100 / [A4 in the first egg])−100. Values greater than zero indicate greater A4 concentration in second than in first eggs within a clutch

able to adjust egg size and content according to annual variation of breeding conditions and as a function of male feet colour, a trait that indicates male condition (Velando et al. 2006) and probably male feeding effort (Velando et al. 2005). After male feet colour was modified to a duller blue, females were able to rapidly adjust egg investment, not only through a decrease of second-egg volume, as found by Velando et al. (2006), but also by decreasing second-egg mass and the relative yolk concentration of A4 in second eggs during a poor year. Furthermore, females mated with experimental males deferred the laying of second eggs. The fact that females adjusted investment in eggs based on a male sexual trait supports the differential investment hypothesis (Burley 1986, 1988; Gil et al. 1999; Cunningham and Russell 2000; Groothuis et al. 2005). The results suggest that females are capable of fine-tuning various egg components depending on prevailing mate and environmental breeding conditions.

By decreasing egg mass and size, females may influence the embryo development, hatching success, size and weight at hatching, chick growth rate and survival (Williams 1994; Cunningham and Russell 2000; Christians 2002; Wagner and Williams 2007). In the blue-footed booby, egg size has a positive effect on hatching success and females seem to reduce their relative investment in second eggs compared to first eggs as the season advance and breeding conditions deteriorate (D'Alba and Torres 2007). In the present study, we found that, compared to eggs produced during a poor year, during a good year females produced heavier and larger first eggs, but relative to the first egg in the clutch, females produced heavier, but not larger, second eggs during a poor year, suggesting that females may vary their investment in eggs of different laying order according to annual breeding conditions. Moreover, when the male feet colour was modified to a duller blue, females decreased size and mass of second eggs. Thus, blue-footed booby females seem to anticipate rearing conditions and adjust investment accordingly using a combination of signals that may directly affect her own condition and access to food, such as annual variations of breeding conditions, and indirect signals, such as the male feet colour, a phenotypic trait that indicates male condition (Velando et al. 2006) and paternal investment (Velando et al. 2005).

In both years of study, females paired with experimental males delayed the laying of the second egg compared to control females. In a similar study, Velando et al. (2006) did not detect such an effect probably because in their study male feet colour manipulation was done 24 to 48 h after the first egg was laid, whereas in the present study, male feet colour was manipulated less than 24 h after the first egg was laid, giving females a longer period to vary investment in eggs and laying dates. The blue-footed booby is a species with aggressive sibling competition and facultative brood reduction (Drummond et al. 1986). The senior chick within a brood hatches on average 4 days before the junior chick, and this asynchrony at hatching is key in determining the output of sibling competition (Osorno and Drummond 1995). Experimental duplication of the hatching interval resulted in poorer growth, 50% increase of aggression and a greater mortality for junior chicks in the experimental group compared to the control group (Osorno and Drummond 1995). Thus, by postponing the laying of the second egg, females paired with males with duller blue feet are probably increasing the competitive asymmetries between brood mates and facilitating brood reduction.

Blue-footed booby females were able to transfer androgens in relation to expected breeding conditions. Females transferred more T to first eggs during a good year than during a year with poor breeding conditions, and for second-laid eggs, the absolute and relative concentration of yolk A4 and the relative concentration of yolk T were higher during a poor year compared to a good year. An increasing number of studies indicate that even minor variations of maternally derived yolk androgens can have important fitness effects on offspring (Schwabl 1996; Groothuis et al. 2005; Rubolini et al. 2006; Von Engelhardt et al. 2006; Tobler et al. 2007). For instance, in the black-legged kittiwake, *Rissa tridactyla*, concentrations in the yolk of A4 and IgGs are positively correlated (Gasparini et al. 2007), and in vitro experiments showed that, contrary to T, A4 enhances immune system (Yao and Shang 2005). Moreover, it has been suggested that higher concentrations of yolk A4 may be related to the production of highly competitive phenotypes in species with communally breed-

ing systems or colonial life (Cariello et al. 2006; Gil et al. 2007). Female condition, which partly depends on environmental breeding conditions, has been shown to influence deposition of androgens in eggs (Verboven et al. 2003; Sandell et al. 2007). Experimental manipulation of food availability previous to egg laying showed that females in good condition reduced the yolk androgen content of their eggs without altering offspring performance (Verboven et al. 2003) and modified the within-clutch pattern of yolk androgen allocation (Sandell et al. 2007). If increasing levels of yolk androgens have a positive effect on blue-footed booby offspring, by transferring more absolute and relative concentrations of A4 and more relative concentration of T into second eggs, females are probably increasing the probability of survival of second-hatched chick during a poor year.

Interestingly, when the feet colour of the mate was modified to a duller blue, females transferred relatively less A4 (but not T) to second eggs than to first eggs within the clutch during a poor year but not during a good year. In our study, first eggs in the control group had significantly more A4 than first eggs in the experimental group during the good year; thus, the interpretation of the results should be taken with caution. In principle, by analysing the relative allocation to first and second eggs within the clutch, we have taken into account this variation, yet more studies will be needed to confirm the results. At present, the results suggest that during a poor year females are probably increasing the probabilities of survival of second chicks by transferring more androgens to second eggs, but when mate feet colour deteriorates during a poor year females decrease the survival probability of second chicks by transferring relatively less A4 to second eggs. In a previous study in the blue-footed booby carried out at the end of an extended El Niño event, thus females were probably in poor condition and ecological prospects for incubating clutches and feeding chicks may also have been poor, no differential allocation of T and DHT according to laying order was detected; yet second eggs received marginally less A4 than first eggs (Drummond et al. 2008). Experimental studies have found that females modified the levels of yolk androgens according to mate attractiveness (Gil et al. 1999, 2004b; Tanvez et al. 2004; Loyau et al. 2007), although others have failed to find such an effect (Rutstein et al. 2004; Marshall et al. 2005). In our study, females were apparently able to rapidly modify the relative allocation of A4 between egg siblings according to a sudden deterioration of male feet colour during a poor year. During a year with poor breeding conditions, females are probably constrained to invest in eggs; hence, varying the yolk concentration of A4 may be an alternative to vary more expensive resources such as lipid-rich yolk component. Adjusting the relative concentration of yolk A4 between siblings may influence sibling asymmetries within a clutch (Schwabl 1997; Sockman et al. 2006; Sandell et al. 2007), which may be an adaptive strategy particularly in a siblicidal bird.

In this study, we show that blue-footed booby females are able to rapidly adjust investment in eggs according to variations in expected breeding conditions and in response to a dynamic trait such as male feet colour. In this species with a long period of parental care, reduction of paternal effort negatively affects the condition and, probably, future reproduction of females (Velando and Alonso-Alvarez 2003). Rearing a brood with a mate in poor condition during a poor breeding year is likely to be costly for females, either because females will have to compensate for a low paternal effort or because the reproductive value of the brood will decrease. Then, under these conditions, adjusting various egg components to facilitate brood reduction may be an adaptive female strategy. Moreover, this study suggests that mate evaluation and breeding decisions continue after pairing; therefore, males should maintain attractive feet colour beyond the courtship period and until laying is complete to assure paternity (Torres and Velando 2003) and to increase female investment in eggs.

Acknowledgment We are grateful to M. Cerbón, A. Córdoba, H. Drummond, D. Gil, A. Velando, K. Renton, two anonymous Referees and J. Graves for helpful comments and discussion during the study, to E. Villaseñor, D. Gonzalez and A. Nava Sánches for their great help during field work and to the Laboratorio de Hormonas Esteroides del Instituto Nacional de Ciencias de la Salud y de Nutrición for logistic support for the androgen determination. The project was supported by the Universidad Nacional Autónoma de México (UNAM, PAPIIT IN211406) and CONACYT (47599). Logistic support was provided by the Armada de México, the staff from the Parque Nacional Isla Isabel and the fisherman from San Blas, Nayarit. The experiments comply with the current laws of Mexico; permissions were granted by SEMARNAT and the Parque Nacional Isla Isabel. During the study, F. Dentressangle was supported by a scholarship for graduate studies from UNAM.

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Male foot color in the blue-footed booby affects hatching success, chicks' androgen concentration and immune response

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Short title: male attractiveness affects chick survival

ABSTRACT

Blue-footed booby females adjust their investment in eggs according to male feet color, a dynamic condition-dependent sexual trait. When male feet color is artificially dulled during laying, females decrease egg mass and volume and yolk androstenedione, suggesting that if apparent condition of mate deteriorates, females may facilitate brood reduction. Here, we investigated the effects of differential investment in eggs on hatching success and chicks´ cellular immune response. First, to assess whether plasma androgens of 7-d old chicks could be used as a proxy of maternal yolk androgens allocation, we tested whether yolk and plasma androstenedione, testosterone and dihydrotestosterone concentrations were correlated. To investigate the effects of differential investment in eggs, male feet color was modified to duller blue after the first egg was laid, and hatching success, fertility of second eggs and chick's cellular immune response (PHA skin test at age 15-d) were recorded. Yolk androstenedione (but not testosterone or dihydrotestosterone) was positively correlated with plasma concentration in chicks and with their cellular immune response. Second eggs in the experimental group had lower hatching success and were fertilized in lower proportion than control eggs. Second chicks fathered by males with duller feet had lower plasmatic androstenedione and dihydrotestosterone concentrations and lower cellular immune response than second control chicks. Thus, differential investment in eggs according to male feet color influenced hatching success and the chick's plasma androgens and cellular immune response. These results suggest that blue-footed booby females may facilitate brood reduction by modifying their investment in eggs when mate feet color suddenly deteriorates.

Keywords: maternal effects, differential maternal allocation, sexual traits, egg fertility, androgens, androstenedione, testosterone, cellular immune response, *Sula nebouxii*.

INTRODUCTION

Female mate choice has traditionally been considered one of the major evolutionary forces responsible for the elaborate male ornaments (review in Andersson 1994). More ornamented males might provide genes for attractiveness or viability, and/or high parental investment (Fisher 1930; Andersson 1994). Therefore, females may use male ornaments as indicators of the expected value of current reproduction (Andersson 1994), and when mated to an attractive male, may adaptively increase reproductive investment (Burley 1986, 1988). Accordingly, females paired with attractive males have been found to fertilized eggs in higher proportion (*Coturnix japonica,* Adkins-Regan 1995; Persaud and Galef 2005) and to adjust their investment in traits that favor offspring survival and development (e.g. egg size, *Anas platyrhyncos*, Cunningham and Russell 2000; *Coturnix chinensis,* Uller et al. 2005; *Taeniopygia guttata* , Gilbert et al. 2006; yolk testosterone, *Taeniopygia guttata*, Gil et al. 1999, 2004; *Serinus canaria*, Tanvez et al. 2004; yolk androstenedione, *Hirundo rustica* Gil et al. 2006; antibodies, *Hirundo rustica,* Saino et al. 2002a; carotenoides, Saino et al. 2002b).

In birds, maternal effects mediated via differential investment in eggs can have a strong impact on offspring fitness, and in some cases lead to adaptive phenotypic plasticity (Mousseau and Fox 1998a and b; Dufty et al. 2002; Lessells 2008). Females transfer not only nutrients to their eggs but also other substances such as antibodies, antioxidants, RNA, and hormones, all of which are thought to influence hatching success, and offspring behavior and development (Williams 1994; Price 1998; Nager et al. 2000; Christians 2002). Particularly, the study of maternally derived yolk hormones and their effects on offspring post-natal growth and development has advanced rapidly (reviews in Gil 2003; Groothuis et al. 2005; Groothuis and Schwabl 2008). Many

studies have investigated the role of yolk androgens, particularly of testosterone (T), and have found, for example, that a high concentration of yolk T, enhances begging behavior and the amount of food received by black-headed gull chicks, *Larus ridibundus* (Eising and Groothuis 2003), and the social rank after fledging of canary chicks, *Serinus canaria* (Schwabl 1993). On the other hand, negative effects of increased yolk T on chick immune response and metabolic rate have also been found (Sockman and Schwabl 2000; Müller et al. 2005; Navara et al. 2005; Rubolini et al. 2006; Von Egelhardt et al. 2006; Tobler et al. 2007).

Recently, it has been suggested that high levels of yolk androstenedione (A4, a precursor of T; Horton and Tate 1966) could be involved in the production of competitive phenotypes in social groups (Cariello et al. 2006; Gil et al. 2007), promote chick growth (Gil et al. 2006), and contrary to T, enhance the immune system (Yao and Shang 2005; Gil et al. 2006). Furthermore, yolk A4 concentrations can vary according to male attractiveness in the barn swallow (*Hirunda rustica*, Gil et al. 2006), and female blue footed boobies (*Sula nebouxii*) decrease yolk A4 (but not T) concentrations when male attractiveness suddenly deteriorates (Dentressangle et al. 2008). Also, high levels of circulating dihydrotestosterone (DHT) are associated with aggressive behavior in black headed gulls chick during the first 15 days of life (Groothuis and Ros 2005). Hence, in addition to T, other yolk androgens such as A4 and DHT may influence the offspring condition and phenotype.

Blue-footed boobies have an extended period of parental care (approximately 5 months, Nelson 1978, Guerra and Drummond 1995; Torres and Drummond 1999), and male contribution to parental care is important for female breeding success (Velando and Alonso-Alvarez 2003). Blue-footed booby females prefer males with bright green-blue feet (Torres and Velando 2003), which reflects better nutritional and health

condition compared to males with duller blue feet (Velando et al. 2006). A cross-fostering experiment of one day old chicks showed that chick's body condition at 15 days correlated with natural male feet color during incubation, suggesting that male feet color could indicate future paternal investment (Velando et al. 2005). Moreover, experimental reduction of paternal effort, one week after chicks hatched, had a strong negative effect on the condition when chicks were 45-50 days, and probably on the future reproduction of females (Velando and Alonso-Alvarez 2003). Therefore, females should adjust their investment in relation to current mate condition.

Accordingly, manipulation of male feet color to duller blue, mimicking males in poor condition, showed that females reduced their copulation rate (Torres and Velando 2003), egg volume (Velando et al. 2006), egg mass, yolk A4 concentration, and increased laying asynchrony (Dentressangle et al. 2008). These results suggest that females whose mate condition apparently deteriorates during egg laying modify their breeding decisions favoring brood reduction. Blue-footed boobies show aggressive sibling competition and facultative brood reduction, frequently leading to the elimination of the second chick when food provisioning is scarce (Drummond and Garcia-Chavelas 1989). Hence, females could favor brood reduction by modifying egg size and composition, or could avoid fertilization by males in poor condition (Persaud and Galef 2005), as sperm expulsion has been observed in this species (Osorio-Beristain M. personal communication).

Here, we investigated whether female differential investment in eggs after a sudden deterioration of male feet color affects hatching success and chicks' androgen concentration and cellular immune response. First, to assess whether plasma concentrations of androgens in young chicks could be used as a proxy of female investment of androgens in the yolk (Sasvári et al. 1999; Gil 2003), we evaluated the

relationship between concentrations of A4, T, and DHT in yolk and plasma of 7-d old chicks. Secondly, to evaluate the consequences for the offspring of female differential investment in eggs according to mate feet color, one day after the first egg was laid, we captured males and modified their feet color to duller blue, mimicking males in low condition, and measured hatching success of second eggs, plasma androgen concentrations of 7-d old second chicks, and their cellular immune response at the age of 15 days. Moreover, unhatched eggs were opened to determine if they were fertilized or not. If female differential investment in eggs when mate feet color deteriorates favors brood reduction we predicted that compared to controls, second eggs in the experimental group will have (1) lower hatching success, and (2) a greater proportion of unfertilized eggs. Additionally, we predicted that chicks fathered by males with duller feet will have (3) lower plasma androgen concentrations at the age of seven days, if yolk and plasma androgen concentrations at this age are related, and (4) lower cell-mediated immune response (CMI response) compared to control chicks, if yolk A4 or plasma A4 concentration stimulate the immune system.

METHODS

The study was carried out in the breeding colony of the blue-footed booby located at Isla Isabel, off the Pacific coast of Mexico (25° 52´N, 105° 54´W). Field work was carried out from January to April 2006 and from January to March 2007.

Yolk samples

As egg yolk biopsies negatively affect hatching success (Dentressangle et al. 2008), we assessed whether androgen concentrations in very young chicks reflect yolk androgen concentrations of maternal origin. This was studied in 2006 together with the potential effects of yolk androgens on chick's CMI response. Less than 24 hours after laying of each egg, a yolk sample (15-20 mg) of 53 eggs (13 two-eggs clutches and 9 three-eggs clutches) was obtained by introducing a syringe (needle type 21G) into the egg through a small hole in the shell (Schwabl 1993). The hole was sealed using a tiny drop of dentistal cement (VIARDEN, Mexico), and immediately after, the egg was placed back in the nest. Yolk samples were stored in liquid nitrogen until laboratory analyses were performed. The procedure of nest monitoring, chick blood sampling at the age of seven days and PHA challenges at the age of 15 days followed the protocol described below.

From the 53 sampled eggs, 15 chicks hatched and 11 reached the age of 7 days. We could not obtain a blood sample from one chick, hence 10 chicks were used for the analysis and all were single chick (eight chicks hatched from the first egg and two from the third one between March 2nd and April 7th).

Experimental modification of male feet color

In 2007, courting pairs were monitored daily to determine laying date. We manipulated feet color of 71 males less than 24 hours after the first egg was laid. Males were captured at night, banded with a numbered metal ring and randomly assigned to either the experimental or the control group. In the experimental group, feet color was modified to a duller blue with a non-toxic make up to mimic a male with low nutritional condition (Torres and Velando 2003). In the control group, we performed the same manipulation but without changing the original male feet color. This method of color modification has been used before with no apparent effects on the bird behavior and the

artificial color on experimental males lasted for 5–6 days (Torres and Velando 2003). Total handling time per bird was less than 5 minutes. Due to technical failure of the spectrophotometer in the field (MINOLTA 2600d), feet color was measured in a sub-sample of males (19 controls and 28 experimentals) before and after the manipulation. Before the manipulation, feet color of control and experimental males did not differ in the peak of maximum reflectance (mean ± SE, 518.8 ± 10.52 nm for control males and 522.5 ± 3.01 nm for experimental males; Mann-Whitney test, $U = 225.5$, $P = 0.73$). After the manipulation, the mean peak of maximum reflectance of experimental males decreased 10.83% (465.9 ± 0.88 nm) compared to males in the control group ($U = 32.0$, $P < 0.001$). Lower values of the peak of maximum reflectance of males in the experimental group remained within the natural range of variation reported for courting males in the same population (Velando et al. 2006). From the 71 captured males, 50 pairs (25 controls and 25 experimentals) established a clutch of two eggs and were used in the experiment.

Nest monitoring and chicks measurements

Nests were inspected daily until the clutch was completed, every two days until day 38 of incubation, and daily thereafter to register the exact hatching date (second eggs mean ± SE incubation period, 40 ± 1.45 days, range = 38.5 to 43.70 days; D'Alba and Torres 2007). All freshly laid eggs (less than 24 hours after laying) were marked with a non-toxic pen and weighted using an electronic balance (± 0.1g; MS500 Pesola Switzerland). Their maximum length and width were measured with a caliper (± 0.1mm) to calculate egg volume in cm^3 (length*width^2*0.51/1000; Hoyt 1979). Eggs that did not hatch after 49 days of incubation were opened and considered unfertile

when there was no evidence of embryonic development (blood spot or embryo) (Adkins-Regan 1995; Persaud and Galef 2005).

At the age of 7 days chicks were sampled for blood (approximately 300µl) from the brachial vein with heparinized capillaries; all samples were taken between 11:00 - 13:00 hrs. Samples were stored on ice and within two hours after collection were centrifuged (8000 rpm during 10 min) to separate the plasmatic fraction from the cellular one. Plasma was stored in liquid nitrogen until hormones assays were performed in the laboratory. The cellular fraction was mixed with a lyses buffer (100mM EDTA, 100mMTris, 2%SDS, pH = 8.0) and stored at ambient temperature for later identification of the sex of the chicks. For DNA extraction (to determine the sex of the chicks), 100µl of NaOH (0.5M) were added to 20µl of blood cellular fraction (stored in lysis buffer) and then heated to 95°C for 5 min then PCR amplification and bands identification followed the technique described in Fridolfsson and Ellegren (1999).

In order to estimate the CMI response, 15-d old chicks were injected subcutaneously in the left wing-web with 0.2mg of fitohemaglutinin (PHA, Sigma, St Louis, USA) in 0.1ml of phosphate buffered saline solution (PBS) (Smits et al. 1999). The point of injection was marked with an indelible marker. Three replicate measurements of the patagium thickness were taken with a digital micrometer (to the nearest 0.001 mm, Mitutoyo) prior to the injection, and again 24 h later. Wing-web thickness measurements were significantly repeatable, both for initial (Pearson correlation, $r > 0.94$, $P < 0.001$) and final measurements ($r > 0.99$, $P < 0.001$). The CMI response was estimated as the change in thickness (mm) of the wing-web 24-h post-injection. To control for chick condition in the analysis of CMI response, 15-d old chicks were weighted to the nearest 0.1g with an electronic balance. From our sample of 50 two-egg clutches, 25 second chicks hatched, 21 from two-chick broods and 4 from

one-chick broods (three from control clutches and one from an experimental clutch). As brood size is known to influence chick's CMI response and plasma T levels (Naguib et al. 2004), and in the blue-footed booby corticosterone levels are associated with the social status of chicks (Nuñez de la Mora et al. 1996), to avoid confounding effects only clutches where both chicks hatched were included in the analyses of plasma androgens and CMI response (n = 10 controls and 11 experimentals).

Hormone Assays

Yolk Androgen concentrations were determined by Radio Immune Assay (RIA) following the protocol described in Dentressangle et al. (2008). Briefly, 10-15 mg of yolk was homogenized in 1 ml of distilled water; 0.5 ml of this homogenized solution was mixed with 5 ml of diethyl ether and vortexed during 1 min. The ether phase was decanted after snap freezing in an alcohol bath at -30ºC and evaporated. The dried extract was redissolved in 1 ml of isooctane and passed through cellite columns for androgen separation. The mean recovery values were 64% for A4, 57% for DHT and 58% for T. Coefficient of variation inter assay was 6.87% for A4, 8.38% for T and 6.91% for DHT, while intra assay variation was 3.07% for A4, 3.24% for T and 3.32% for DHT.

Plasma A4, T and DHT concentrations in chicks were also determined by RIA following the procedure described in Salame-Mendez et al. (1998) and previously validated in humans and sea turtles with recoveries for all androgens above 80%, (the laboratory was certified according to the quality norm ISO 9001:2000 for androgens analysis). Briefly, for androgen extraction 0.1 - 0.2 ml of chick plasma was used and directly mixed with 5 ml of diethyl ether and vortexed during 1 min. Then A4, DHT and T were separated through celite chromatography columns, using 3.5 ml of isooctane

(100%), 3.5 ml of isooctane and ethyl acetate (95%:5%), and 3.5 ml of isooctane and ethyl acetate (85%:15 %), respectively. The rest of the technique followed the standard procedure of RIA (Salame-Mendez et al. 1998). All plasma samples were run in a single assay and coefficients of variation intra assay were 3.26% for A4, and 2.86% for DHT. Specific A4 and DHT antibodies were provided by MP Biomedicals, LLC, Ohio, USA (catalogue number; A4: 61320 and DHT: 61340). T antibodies were provided by World Health Organization RIA Reagent Program (catalogue number: K200710). The cross-reactivities of the antibodies were A4:T = 4.5%, T:A4 = 3.5%, T:DHT = 1.3%, DHT:A4 = 2.4%, DHT:T = 22.7%. DHT was not detected in 3 blood samples meaning the concentration was between 0 and 6.5 pg/ml (the minimum range of detection) thereby we attributed the conservative value of 3.5 pg/ml to those samples.

In 2007, after separation though cellite columns, T concentrations in chick plasma were determined with the automated chemiluminescent immunoanalyzer IMMULITE® (Diagnostic Products Corporation, Los Angeles, USA) with Siemens kits (catalog number LKTW1). This technique does not use radioactive products and allow us to obtain results faster than with the standard RIA. The sensitivity and precision of this technique are the same used in the standard RIA (Immulite® for total testosterone 2005), and results obtained showed 4.6 % of variation compared to RIA (internal report for the international quality control program of Buenos Aires XXI, CEMIC). Intra assay coefficient of variation was 6.56%.

Statistical analysis

To evaluate whether yolk and chick plasma androgen concentrations were correlated we used Pearson correlations. Hatching success was analyzed using Generalized Linear Models with binomial error distribution (PROC GENMODE; SAS Institute 1999). The

initial model included treatment, second egg mass and hatching success of the first egg as factors and all 2-way interactions with treatment. Laying date of second eggs was slightly earlier (mean ± *SE*, 3 ± 0.49 days) in the control group than in the experimental group, however the difference was not significant ($F_{1,48} = 3.72$, $P = 0.06$). Laying date was initially included in the analyses but was not significant (all $P > 0.17$), then this variable was excluded from the models. The proportions of unfertilized eggs in the control and the experimental group were compared with a Chi-square test.

Egg mass and volume, chick plasmatic androgen concentrations and CMI response were analyzed using General Linear Models with normal error distribution. For the analyses of second egg mass and volume initial models included as factors treatment, hatching date and first egg mass or volume, respectively, and all 2-way interactions with treatment. Plasma T concentration was correlated with A4 and DHT (Pearson correlations, A4 $r = 0.403$, $P = 0.020$; DHT $r = -0.494$, $P = 0.003$); thus, to avoid coliniarity independent models for each androgen were performed. For the analyses of chick plasma androgen concentrations, initial models included treatment, hatching date, and chick sex as factors and all 2-way interactions with treatment. For the analyses of CMI response, initial models included treatment and chick sex as factors plus their interaction, and A4, T or DHT and chick body mass at the age of 15-d as covariates. In all analyses final models were obtained using a backward elimination procedure (Crawley 2005). First, the interaction terms were sequentially removed from the full model when the variance explained did not significantly improve the model ($\alpha = 0.05$). After all two-way non-significant interactions were removed from the model, the same deletion procedure was performed with the main effects of the predictor variables (Crawley 2005). Mean ± SE are shown throughout the manuscript.

RESULTS

Relationship between yolk and plasma androgen concentrations

Concentrations of yolk A4 and plasma A4 from 7-d old chicks hatched from yolk sampled eggs in 2006 were positively correlated (Pearson correlation $r = 0.84$, $P = 0.002$, $n = 10$ chicks; Fig. 1a). Yet, no correlation between yolk T and plasma T ($r = 0.32$, $P = 0.51$), or yolk DHT and plasma DHT from 7-d old chicks was found ($r = 0.10$, $P = 0.77$). Yolk A4 concentration correlated positively with the CMI response of 15-d old chicks ($F_{1,8} = 15.62$, $P = 0.004$; Fig. 1b). No effects of yolk T ($F_{1,8} = 2.14$, $P = 0.18$), yolk DHT ($F_{1,6} = 0.009$, $P = 0.92$) or chick sex (in all models $P > 0.31$) on chick CMI response were detected.

Experimental modification of male feet color

Egg mass, egg volume and laying interval

First eggs (i.e. eggs laid before the manipulation) from the experimental and control group did not differ in mass ($F_{1,47} = 2.54$, $P = 0.11$) or volume ($F_{1,48} = 0.21$, $P = 0.64$). The experimental manipulation of male feet color did not have an effect on the second egg mass (treatment $F_{1,46} = 0.11$, $P = 0.73$, treatment*mass of the first egg $F_{1,44} = 1.71$, $P = 0.19$) or volume ($F_{1,47} = 0.22$, $P = 0.63$, treatment*volume of the first egg $F_{1,45} = 0.25$, $P = 0.61$). Laying date of second eggs had no significant effect on second egg mass ($F_{1,45} = 0.02$, $P = 0.88$) or volume ($F_{1,46} = 0.21$, $P = 0.64$). The mass and volume of second eggs were positively related to the mass and volume of the first eggs in the

clutch, respectively (mass of the first egg $F_{1,47} = 49.52$, $P < 0.001$; volume of the first egg $F_{1,48} = 57.04$, $P < 0.001$). Laying intervals between first and second eggs of control (5.16 ± 0.40 days) and experimental clutches did not differ (4.56 ± 0.48 days; Mann-Whitney test, $U = 240.5$, $P = 0.16$).

Hatching success and sex ratio

Hatching success of second eggs was on average lower in the experimental group (56%) than in the control group (64%; $\chi^2_{1,45} = 4.35$, $P = 0.037$), even after the effect of the mass of the second egg was considered (egg mass $\chi^2_{1,45} = 5.57$, $P = 0.018$, treatment*egg mass $\chi^2_{1,45} = 4.20$, $P = 0.040$). Hatching success decreased with increased egg mass in the control group, whereas little variation with egg mass was found in the experimental group (Fig. 2a). The hatching probability of second eggs increased when the first egg in the clutch hatched ($\chi^2_{1,45} = 15.65$, $P < 0.001$). From the eggs that did not hatch, the proportion of eggs that were unfertilized (i.e. eggs that did not have any signs of blood spot or embryo) was higher in the experimental group (63.63% out of 11 unhatched eggs) than in the control group (33.33% out of 9 unhatched eggs; $\chi^2_{1,19} = 4.54$, $P = 0.03$; Fig. 2b).

The sex ratios of second chicks that hatched in the experimental group (64%, 7 females out 11 chicks) and in the control group (40%, 4 females out 10 chicks) were not significantly different ($\chi^2_{1,20} = 2.91$, $P = 0.087$).

Androgen concentrations

Experimental manipulation of male feet color during egg laying had a significant effect on plasmatic A4 and DHT concentrations of 7-d old chicks (A4, $F_{1,19} = 4.52$, $P = 0.047$; DHT, $F_{1,19} = 7.56$, $P = 0.01$, interactions with treatment all $P > 0.31$). Experimental

chicks had on average 22.51% less A4 and 39.62% less DHT than control chicks (Fig. 3a and b); yet, no effect of treatment on plasmatic T concentration was detected ($F_{1,19}$ = 0.05, P = 0.82; Fig. 3c). Second chicks hatched later in the season had higher A4 and T concentration and marginally higher DHT concentration (A4: $F_{1,19}$ = 9.04, P = 0.007; T: $F_{1,19}$ = 13.45, P = 0.002; DHT: $F_{1,19}$ = 4.29, P =0.053, interactions of treatment * hatching date all P > 0.31). Male and female 7-d old chicks did not differ in plasma concentration of A4, DHT or T (main effects: all P > 0.34; interactions of treatment * sex: all P > 0.44).

Cell mediated immune response

The experimental manipulation of male feet color had a significant effect on the CMI response of offspring ($F_{1,19}$ = 8.91, P = 0.007). Chicks from the experimental group had a CMI response 37.43% lower than chicks in the control group (Fig.4). No differences between male and female chicks were detected (all P > 0.47). The chick's CMI response was not related to variation in the plasma concentration of A4 (all P > 0.26), DHT (all P > 0.13), T (all P > 0.22), and chick body mass (all P > 0.18).

DISCUSSION

This study suggests that differential allocation of blue footed booby females to eggs according to mate feet color have strong consequences on parameters that are linked to chick survival and fitness. There was a positive correlation between concentrations of yolk A4 and plasma A4, suggesting that plasma concentrations of androgens during the first days of life may reflect female A4 investment in the egg (Sasvári et al. 1999; Gil

2003). Furthermore, concentrations of yolk A4 were positively related to the CMI response of 15-d old chicks, indicating that the effects of maternal A4 on chick immunocompetence during early life (Apanius 1998). The lack of correlation between yolk T and DHT with their respective plasmatic concentrations suggest that chicks may produce these androgens endogenously (Godsave et al. 2002), independently of the amount deposited in eggs and/or convert A4 into T during early life (as A4 is a precursor of T; Horton and Tate 1966). Thus, variation in the deposition of A4 in the yolk may affect the cellular immune response of chicks, a potentially important trait for chick survival.

In the present study, experimental females did not reduce second egg size (mass and volume), nor did they increase laying interval, contrary to what was found in two studies where male feet color was modified to duller blue (Velando et al. 2006; Dentressangle et al. 2008). It has been shown that females modify their investment in eggs (mass and yolk A4) according to environmental conditions between years (Dentressangle et al. 2008) and to laying date within the same breeding season (D'Alba and Torres 2007). Our sample was composed uniquely of early breeders (in the first quarter of the breeding season unlike Velando et al. 2006 and Dentressangle et al. 2008 studies that were conducted during the first half of the breeding season), thus, it is possible that early breeding females use different strategies to adjust their investment in eggs according to male feet color, by modifying parameters other than egg size or laying interval (see below).

Though no differences in egg size or laying interval were detected, hatching success in experimental second eggs was 8% lower than controls. Additionally, hatching probability of second eggs increased in clutches where the first egg hatched, implying differences on parental quality, such as fertility, nesting site and/or incubation

abilities (Kim and Monaghan 2005). The difference in hatching success of control and experimental eggs could come about if females bias the probability of an egg of being fertilized and/or by differential female investment in the egg composition or incubation behavior (Adkins-Regan 1995; Nager et al. 2000; Royle et al. 2001; Persaud and Galef 2005). Alternatively, the feet color manipulation could have influenced the sexual and incubation behavior of experimental males with negative effects on hatching success; however, this is unlikely because during courtship the same manipulation did not affect the courtship behavior of males (Torres and Velando 2003). In the blue footed booby females vary egg size and the concentration of yolk A4 according to male feet color (Velando et al. 2006, Dentressangle et al. 2008). Hence, they may be able to vary other parameters such as lipids and water content, carotenoides or antibodies that influence the embryo survival. Interestingly, the proportion of infertile second eggs was higher in the experimental group than in the control group, suggesting that females have control on the fertilization of their eggs (Stockley 1997). In this species, when male feet color was manipulated to a dulled blue during courtship, females decreased copulation rate (Torres and Velando 2003), and sperm ejection has been observed (Osorio-Beristain M. personal communication). Although we are unable to identify all the causes of hatching failure, the decrease of hatching success and the increase in the proportion of unfertilized eggs in experimental clutches suggest that females attempted to avoid future compensation costs due to low expected paternal investment during the rearing period (Velando et al. 2005).

Oddly, the probability to hatch of second eggs decreased with egg mass in the control group. In the blue footed booby hatching probability of first and second eggs increases with egg size (D'Alba and Torres 2007). Yet, a study carried out in the same colony found that the probability of hatching increased with egg mass up to eggs of 55g,

and then decreased for heavier eggs (Garcia Peña 2005). Eggs heavier than 55g represented 76% of the eggs in our control group. Thus, egg size and hatching success may not always show a linear relationship, as extremely sized eggs may have lower hatching success (Kontiainen et al. 2008). Hence, egg size does not necessary reflect egg quality (Nager et al. 2000; Clifford and Anderson 2002).

Chicks fathered by males with dulled feet had lower concentrations of plasma A4 and DHT at the age of 7 days, and lower concentrations of plasma A4 are related to lower concentrations of yolk A4. In birds, high levels of yolk A4 are related to the production of competitive phenotypes in social groups (Cariello et al. 2006; Gil et al. 2007); In the guira cuckoos (*Guira guira*), a communality breeding species, eggs with high levels of A4 are those producing chicks with the highest survival probability (Cariello et al. 2006), and in the barn swallow yolk A4 stimulate chick growth (Gil et al. 2006), while high levels of plasma DHT are associated with aggressive behavior in black headed gulls chicks (Groothuis and Ros 2005). In the blue footed booby, hatching asynchrony facilitates the establishment through aggressive behavior of a dominant–subordinate relationship in which typically, the senior chick out competes the younger chick, resulting in differential mortality of the subordinate nestling (Drummond et al. 1991; Osorno and Drummond 1995). Nonetheless, T levels during the first two weeks of life do not seem to be involved in the control of aggressive behavior among siblings in this species (Ramos Fernandez et al. 2000). Furthermore, Drummond et al. (2008) rejected the hypothesis that blue footed booby females facilitate brood reduction by differential provisioning of androgens to eggs of different hatching order, as first and second eggs, collected in early clutches during an Niño event, did no differ in yolk T or DHT concentrations; albeit a marginal non significant decrease in the concentrations of A4 with laying order was detected. However, a recent experimental study suggests that

when the male feet color deteriorates during a year with poor breeding conditions, females decrease yolk A4 concentration with laying order (Dentressangle et al. 2008). Hence, it is likely that differential provisioning of androgens to eggs of different hatching order depend on expected breeding conditions. Future studies should investigate whether lower concentrations of plasmatic A4 and DHT decrease aggression and/or begging behavior in chicks, two key factors in this facultative siblicidal species (Drummond et al. 1991). The fact that 7-d old experimental chicks had lower concentrations of plasma A4 supports previous findings suggesting female ability to decrease yolk A4 concentrations when male feet color experimentally deteriorates (Dentressangle et al. 2008).

The weaker CMI of chicks fathered by a duller footed male may be associated to the lower investment of yolk A4 by females (Dentressangle et al. 2008). However, in our experiment the lack of correlation between the chicks´ CMI response and the plasma A4 concentration suggest that other variables may be involved. Prenatal exposure to androgens has been shown to affect the chick's CMI response (Martin 2000; Muller et al. 2005; Navarra et al. 2005, 2006). *In vitro* experiments showed that A4 stimulated the proliferation of rat thymocytes induced by a mitogen (Yao and Shang 2005), and the thymus is the primary lymphoid organ that generates functionally mature antigen specific T cells (Santoni et al. 2000). Additionally, it has been reported that barn swallow females adjust their investment in yolk antibody concentrations according to male attractiveness (Saino et al. 2002a). A decrease in antibody concentration decreases the frequency of MHC class II cells that are responsible for the presentation of antigens to T cells (Yasuda et al. 1998). Hence, besides the reduction in yolk A4, females may adjust other components of the egg, such as yolk antibodies (not measured in this study), in response to the apparent deterioration of male condition that may depress the

chicks´ CMI response (Yasuda *et al*. 1998; Saino et al. 2002a), with potential negative effects on the chicks´ probability of survival (Muller et al. 2005).

This study supports the idea that maternal effects are a powerful mechanism in shaping the chick's phenotype and possibly in modulating its survival probability (Mousseau and Fox 1998a and b; Dufty et al. 2002; Lessells 2008). Our results indicate that when the reproductive value of the clutch decreases due to poor partner's condition, female blue footed boobies may strategically facilitate brood reduction by modifying a set of parameters that affect the offspring probability of survival (Velando et al. 2006; Dentressangle et al. 2008).

Acknowledgments

We are grateful to M. Cerbón, A. Córdoba-Aguilar and H. Drummond, for helpful comments and discussion during the study, to J. Morales for valuable comments on the manuscript, to E. Villaseñor, J. Morales, N. Lifshitz, M. Hernandez, A. Ramos for their great help during field work, and to the Laboratory of steroid hormones of the "Instituto Nacional de Ciencias de la Salud y de Nutrición" especially to L. Boeck and R. Chavira for androgens determination. The project was supported by the Universidad Nacional Autónoma de México (UNAM, PAPIIT IN211406) and CONACYT (47599). Logistic support was provided by the Armada de México, the staff from the Parque Nacional Isla Isabel and the fisherman from San Blas, Nayarit. The experiments comply with the current laws of Mexico, permissions were granted by SEMARNAT and the Parque Nacional Isla Isabel. During the study F. Dentressangle was supported by a scholarship for graduate studies from UNAM.

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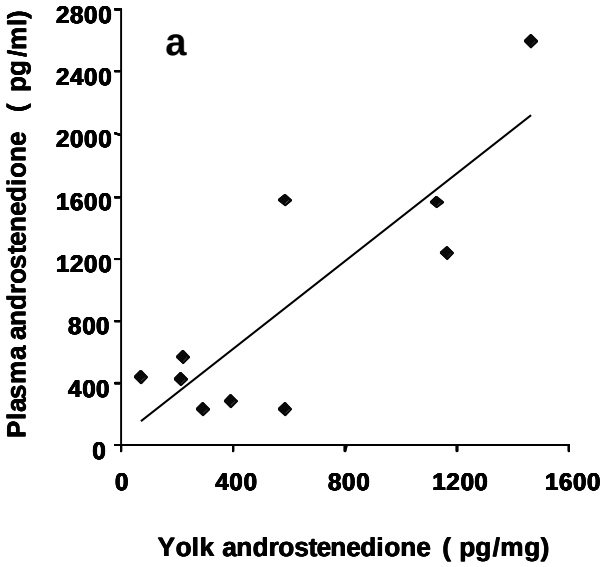
Figures legend

Figure 1. Correlation between yolk androstenedione (A4) concentration (pg/mg of yolk) and (a) plasma A4 concentration of 7-d old chicks (pg/ml), and (b) wing web thickness increase (mm) induced by a PHA challenge in 15-d old chicks.

Figure 2. (a) Hatching probability of second eggs from 25 experimental and 25 control clutches. The adjusted curves were estimated as the residuals of hatching probability of second eggs controlled by hatching success of the first egg. (b) Proportion of unfertilized eggs expressed as the percentage of eggs that did not hatch. In the experimental group male feet color was modified to a dull blue.

Figure 3. Mean (± standard error) plasma concentrations (pg/ml) of (a) androstenedione, A4, (b) dihydrotestosterone, DHT, and (c) testosterone, T, from 7-d old chicks from the control (n = 10) and experimental (n = 11) groups. * $P < 0.05$.

Figure 4. (a) Cell-mediated immune (CMI) response expressed as the increase in wing-web thickness (mm) in response to PHA skin test in 15-d old chicks from the control and the experimental groups. * $P < 0.05$.



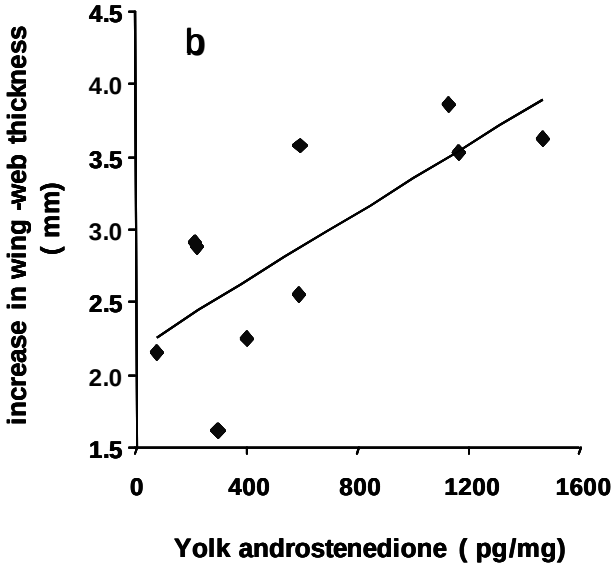
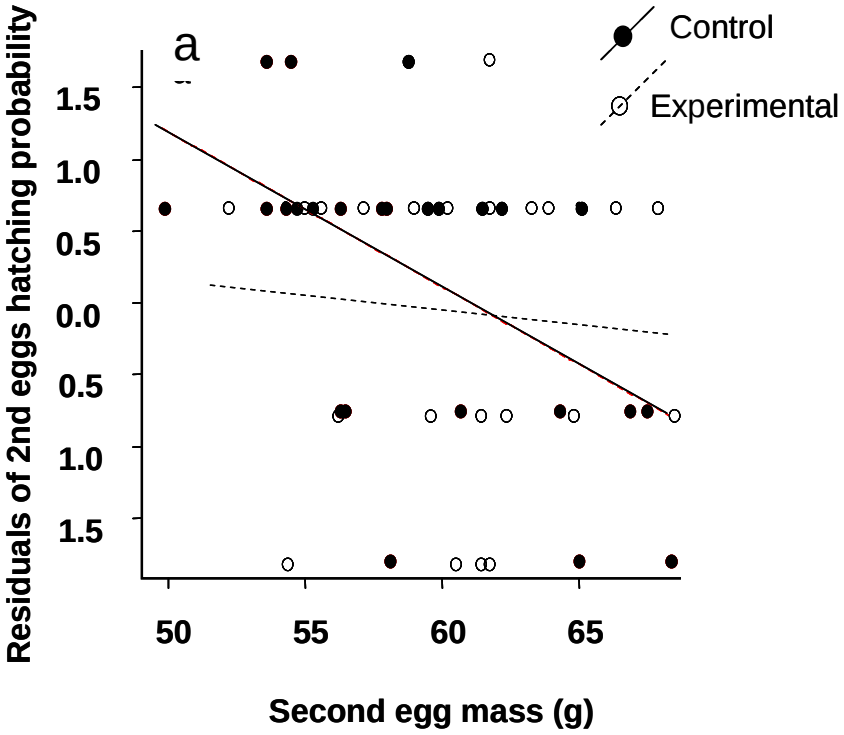


Figure 1a and 1b



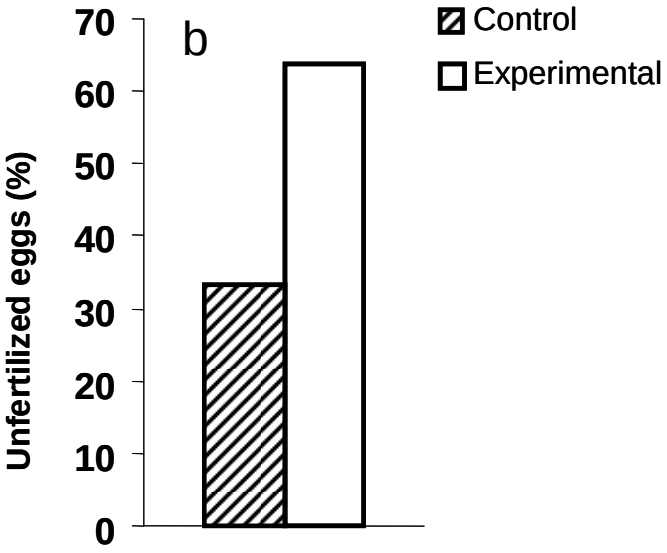


Figure 2a and 2b

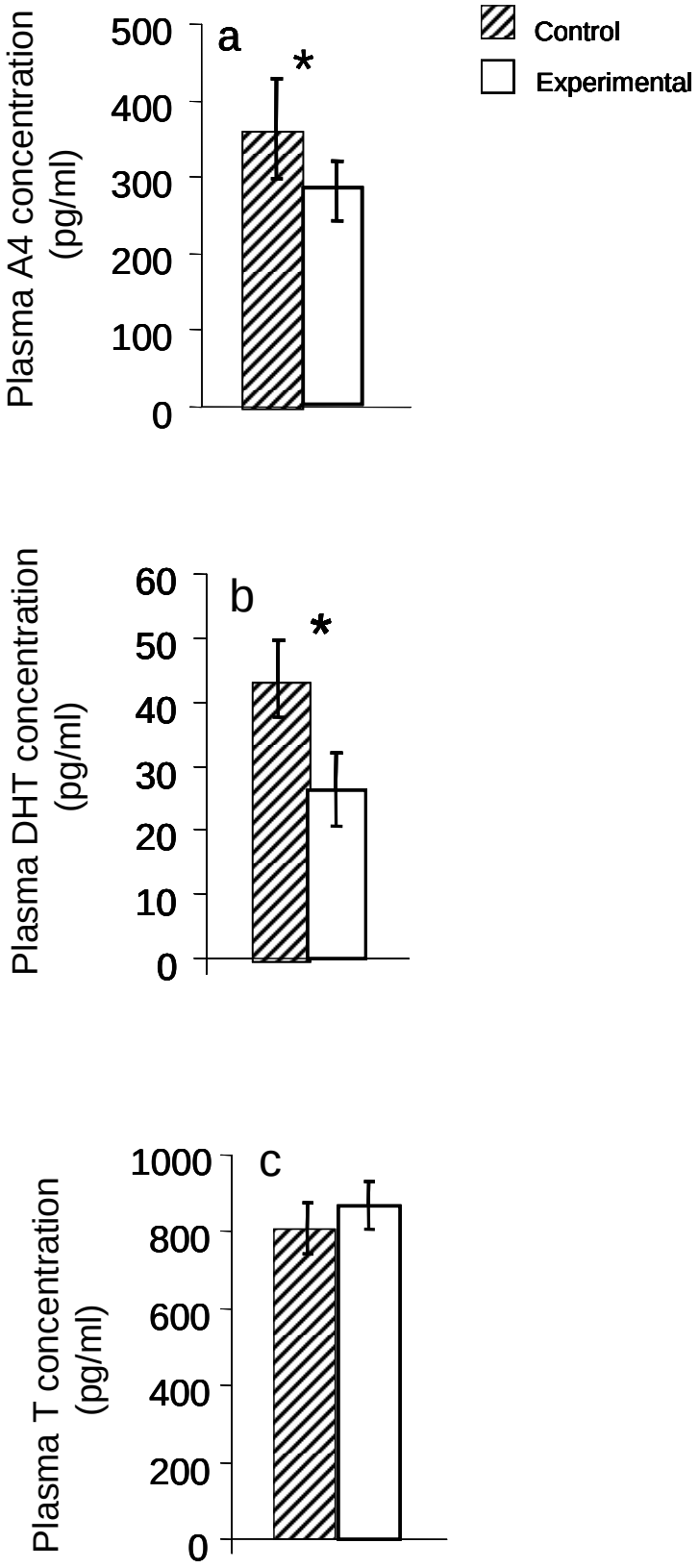

Plasma A4 concentration (pg/ml)
500
400
300
200
100
0
a
*
Control
Experimental
Plasma DHT concentration (pg/ml)
60
50
40
30
20
10
0
b
*
Plasma T concentration (pg/ml)
1000
800
600
400
200
0
c

Figure 3a, b, c

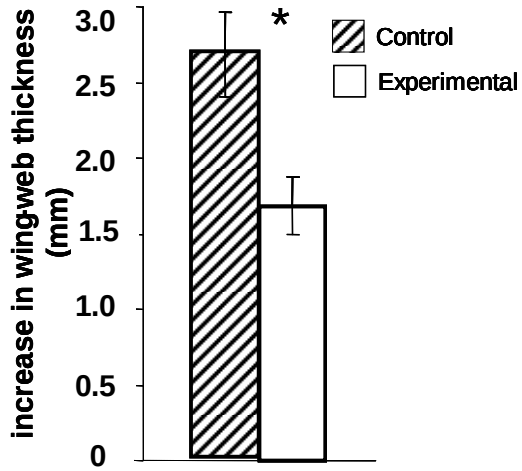

increase in wing-web thickness (mm)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Control
Experimental

Figure 4

Chick phenotype is affected by female differential allocation according to male feet colour in the blue footed booby

Fabrice Dentressangle & Roxana Torres

Abstract

When male ornaments are a reliable indicator of its future rearing contribution, females may adaptively prepare their progeny for the expected conditions they will face through differential resource allocation in eggs. Blue-footed booby females adjust their investment in eggs according to male feet colour, a dynamic condition-dependent sexual trait. Previous results suggest that when the apparent condition of the mate deteriorates, females may facilitate brood reduction. Hence to investigate whether female differential investment in eggs according to male attractiveness affect chick competitive abilities (begging and aggression) and growth rate we modified male feet colour to a dull blue after the first egg was laid and recorded second chicks' behaviour at 10d. Experimental chicks spent less time begging than controls, but no differences in growth rate were found. Aggression was higher in experimental than control chicks, and was positively related to plasma androstenedione concentration, moreover female chicks were 4 times more aggressive than males. Hence, chicks' competitive abilities are affected by female differential allocation according to male attractiveness, and may under poor rearing condition favour brood reduction.

Key words: Begging, aggression, growth rate, androstenedione, maternal effects

INTRODUCTION

Maternal effects may be a strategy to optimize offspring fitness according to the environment the mother predicts her offspring will encounter (Mousseau & Fox 1998). Mate attractiveness could be an environmental factor influencing offspring phenotype through maternal effects, when mothers paired with attractive males increase their investment in the offspring (differential allocation hypothesis; Burley 1986, 1988). Accordingly, in some bird species females paired with attractive males lay larger eggs (Cunningham and Russell 2000, Gilbert et al. 2006, Velando et al. 2006, Dentressangle et al. 2008), larger clutches (Uller et al. 2005), and deposit greater amounts of yolk androgens (Gil et al. 1999, Tanvez et al. 2004, Loyeau et al. 2007, Dentressangle et al. 2008) and antibodies (Saino et al. 2002) than females paired with less attractive males. This differential allocation in eggs can have a strong influence on chick development (Christian 2002, Balthazart et al. 1996, Gil 2003, Groothuis et al. 2005, Carere and Balthazart 2007). In particular, the presence of substantial levels of maternal androgens in the eggs has received much attention because this suggests that mothers may adaptively control individual differentiation in their offspring by differential allocation of these hormones.

Yolk steroids can influence several parameters involved in the competitive abilities of chicks (review in Gil 2003, Groothuis et al. 2005). For example, in the canary, *Serinus canaria*, yolk testosterone stimulates begging behaviour of chicks, and also has a positive effect on the social rank occupied after fledging (Schwabl 1993, 1996). Similar results have been reported for black headed gull chicks, *Larus ridibundus*, in which yolk testosterone stimulates begging, and thus has a positive effect on the amount of food received (Eising and Groothuis 2003) and growth rate (Eising et al. 2001). Additionally, in the house sparrow (*Passer domesticus*) and the black headed gull, exposition to yolk steroids has been shown to affect competitive abilities and the expression of ornaments during adulthood (Strasser and

Schwabl 2004, Eising et al. 2006). In the house sparrow, chicks hatched from testosterone (T) treated eggs showed a larger badge (a trait under sexual selection) and obtained and defended more efficiently food resources (Strasser and Schwabl 2004). Similarly, in the black headed gull the development of the nuptial moult was accelerated by high levels of yolk steroids (Eising et al. 2006). These parameters are important for mate selection and are positively related with reproductive success (Strasser and Schwabl 2004, Eising et al. 2006). Thus, yolk androgens are probably an important factor influencing individual phenotypic variation. Nevertheless, the effects of androgen exposition during embryonic development and early stages of life can differ due to the organizational and activational properties of androgens. For example black headed gull chicks hatched from T injected eggs begged more intensively (Eising et al. 2001), but implanting of T 6 days after hatching provoked a significant decrease in begging behaviour (Groothuis and Ros 2005).

The blue footed booby is a socially monogamous long lived marine bird with a modal clutch size of 2 eggs and biparental care (Nelson 1978). In this species chicks compete aggressively for food and generally the senior chick (hatched 4d earlier than its younger sibling) dominates the junior chick through pecking and biting (Drummond et al. 1989). Under conditions of poor food supply aggression between chicks increases greatly and can lead to the elimination of the last hatched chick (Drummond et al. 1989). Female blue footed boobies prefer males that have green-blue feet compared to males with dulled blue feet (Torres and Velando 2003). Feet colour seems to be an honest signal that reflects both the nutritional status and immune capacity of the individual (Velando et al. 2006) and has been suggested to be an indicator of male future paternal investment (Velando et al. 2005). Male participation in chick rearing is important for female fitness, and a reduction of paternal care results in a reduction in female body mass that can have negative repercussions for future reproduction (Velando and Alonso-Alvarez 2003). Additionally, when male feet colour was

experimentally modified to a dull blue (simulating a male in poor condition) after the first egg was laid females laid smaller eggs (Velando et al. 2006, Dentressangle et al. 2008), increased laying asynchrony, and under poor environmental conditions decreased yolk A4 concentration of second eggs compared to the first egg in the clutch (Dentressangle et al. 2008). Second eggs fathered by experimental unattractive males had lower hatching success and chicks that hatched had on average 22.51% less A4 and 39.62% less DHT than control chicks at 7d (mean ± SE; A4: controls 363.28 ± 65.28 pg/ml; experimentals 281.86 ± 34.47 pg/ml; DHT: controls 43.65 ± 5.92 pg/ml; experimentals 26.36 ± 5.84 pg/ml), and a lower cellular immune response at 15d (Dentressangle and Torres, in prep). Yet, no differences in testosterone concentration were detected between control and experimental second chicks (controls 807.00 ± 67.09 pg/ml and experimentals 868.09 ± 61.11 pg/ml) These results suggest that when the reproductive value of the clutch decreases, due to a partner in poor condition and/or poor environmental conditions, females may strategically favour brood reduction (Velando et al. 2006, Dentressangle et al. 2008, Dentressangle and Torres, in prep).

Here, after the first egg in the clutch was laid we modified feet colour of blue footed booby males to a dulled blue, mimicking a male in poor condition, to investigate whether maternal effects due to male attractiveness influence chick competitive abilities (begging and aggressive behaviour) during the two first week of life, a key period for the establishment of the dominant-subordonate hierarchy (Valderrabano-Ibarra et al. 2006) and growth rate. We take advantage of an experiment in which the same manipulation of male feet colour was performed, in this experiment we found that plasma A4 concentration of 7d old chicks could be used as a proxy of yolk A4 concentration, as these two variables we strongly correlated (Dentressangle and Torres, in prep). If female investment in the second egg when paired with a male in poor condition after laying the first egg influences the competitive abilities of second chicks we predict that second chicks fathered by a male manipulated so as to appear in

poor condition would beg less intensively, be less aggressive and grow slower than control chicks. Moreover, if these parameters are under androgen control (Schwabl and Lipar 2000, Groothuis and Ros 2005, Eising et al. 2006) we predict a positive relationship between plasma chick steroid concentrations at 7 days and begging behaviour and aggression at 10 days and growth rate during the first 15 days of life.

METHODS

The study was carried out at the blue-footed booby breeding colony of Isla Isabel, off the Pacific coast of Mexico (25° 52´N, 105° 54´W), during the breeding season from January to March 2007.

Experimental modification of male feet colour

Courting pairs were monitored daily to determine laying date. We manipulated feet colour of 71 males less than 24 hours after the first egg was laid. Males were captured at night, banded with a numbered metal ring and randomly assigned to either the experimental or the control group. In the experimental group, feet colour was modified to a duller blue with a non-toxic make up to mimic a male with low nutritional condition (Torres & Velando 2003). In the control group, we performed the same manipulation without changing the original male feet colour. This method of colour modification does not influence bird behaviour and the artificial colour on experimental males lasts for 5–6 days (Torres & Velando 2003). Total handling time per bird was less than 5 minutes. Due to technical failure of the spectrophotometer (MINOLTA 2600d), male feet colour was measured in a sub-sample (N = 47, 19 controls and 28 experimentals) before and after the manipulation. Before the manipulation feet colour of control and experimental males did not differ in the peak of

maximum reflectance (mean ± SE, 518.8 ± 10.52 nm for control males and, 522.5 ± 3.01 nm for experimental males; Mann-Whitney test, $U = 225.5$, $P = 0.728$). After the manipulation, the peak of maximum reflectance of experimental males decreased 10.83% (465.9 ± 0.88 nm) compared to males in the control group ($U = 32.0$, $P < 0.001$). Lower values of the peak of maximum reflectance of males in the experimental group remained within the natural range of variation reported for courting males in the same population (Velando *et al.* 2006). From the 71 captured males, 50 pairs ($N = 25$ control and $N = 25$ experimental) established a clutch of two eggs and were used in the experiment.

Nest and chick monitoring

Nests were monitored daily from the 38th day of incubation until hatching to register the exact hatching date (second eggs' mean incubation period 40 ± 1.45 days, range = 38.5 to 43.70 days; D'Alba & Torres 2007). Chicks were weighted at hatching and at day 15 with an electronic balance (Pesola MS500) to the nearest 0.1g. Growth rate during the first 15 days was calculated as: (body mass at day 15 – body mass at hatching) / body mass at hatching. A blood sample (approximately 300µl) was taken with heparinized capillaries from the brachial vein of seven day old chicks; all samples were taken between 11:00 and 13:00 hrs. Blood samples were stored on ice and centrifuged within 2 hours following their collection to separate plasma from cellular fraction. Plasma samples were stored in liquid nitrogen until their analysis in the laboratory to determine A4, DHT and T concentrations by radioimmunoassay (RIA) as described in Dentressangle and Torres (in prep). The cellular fraction was mixed with a lyses buffer (100mM EDTA, 100mMTris, 2%SDS, pH = 8.0) and stored at ambient temperature for later identification of the sex of the chicks using molecular techniques (Fridolfsson & Ellegren 1999).

Behavioral data

From the original sample of 50 clutches, 21 two-chick broods whose second chick was still alive at the age of 10 days were used for behavioural observations (10 controls and 11 experimentals). The sex ratio of first and second chicks was respectively 30% and 40% females in the control group and 45% and 64% in the experimental group, but were not significantly different (first chicks: $\chi^2_{1,20} = 0.07$, $P = 0.78$; second chicks: $\chi^2_{1,20} = 2.91$, $P = 0.087$, Dentressangle and Torres, in prep). Behavioural observations were carried out when the second hatched chick in the brood was 10 days old. Each brood was observed for 3 consecutive hours (7:00 to 10:00h or 15:00 to 18:00h). Half of the control and experimental broods were observed in the morning and half in the afternoon to avoid any possible effect of the time of the day. Ten minutes before the observation began, both chicks in the nest were marked on the head with a coloured dot, weighted with a spring balance (first hatched chicks were weighted with a Pesola 500 ± 5g and second hatched chick with a Pesola 300 ± 2g) and placed back into the nest. At the end of the observation both chicks in the brood were weighted, and the difference between the final and initial mass was used as a proxy of the quantity of food received during the three hours of observation.

Behaviour was recorded by an observer at approximately 5-8 m from the focal nest (boobies are exceptionally tolerant to human presence; Drummond and Osorno 1992). Aggressive behaviours between broodmates were registered continuously as the number of pecks (frequency) and defined as any rapid movement of the head ending in contact of the beak with the other chick, whether resulting in a simple impact or seizing (Drummond and Osorno 1992). Additionally, during each period of 30s (signalized by a bleep) the observer recorded whether each chick was inactive (the whole period with the head resting on any substrate or invisible under the attending adult) or active (Drummond et al. 2003), and if active, whether it begged for food during this period (Villaseñor and Drummond 2007). Begging was recorded when a strong or weak nodding (bill tip oscillations), accompanied by

jabbing or not, occurred (Villaseñor and Drummond 2007). During begging, chicks generally vocalize, nevertheless we did not register this behaviour as it was difficult to identify precisely which chick was calling.

Statistical analysis

Plasma T concentration was correlated with A4 and DHT (Pearson correlation, A4: r = 0.403, $P = 0.020$; DHT: r = -0.494, $P = 0.003$), therefore to deal with colinearity, for each depend behavioral variable three separate models were constructed including only one androgen at a time.

The mass at hatching and growth rate during the first 15 days of second hatched chicks were analyzed with General linear Models with normal error distribution. The models included treatment, sex, A4, DHT or T, first chick mass at hatching or growth rate and the interaction of first chick growth rate or mass at hatching and treatment. Similar results were found when 15-d body mass and 15-d ulna length of second chicks were analyzed, thus only results of mass at hatching mass and growth rate during the first 15 days analyses will be presented.

We analyzed active time, defined as the number of bouts of 30 s during which a chick was active with General Linear Models with normal error distribution, the proportion of active time spent begging (hereafter begging) with Generalized Linear Models with a quasibinomial distribution to correct for overdispersion, and the frequency of aggressions with Generalized Linear Models with Poisson distribution and the Pearson Chi square correction for overdispersion (Crawley 2007). The behaviour of first chicks was only compared between treatments. For the analyses of second chick behaviour, the initial models included treatment, chick sex, the behaviour (active time, begging or the frequency of aggression) of the first chick and A4, T or DHT and their two way interactions, and the

change in body mass of the second chick during the observation as covariate. Small differences in sample sizes among analyses are due to the exclusion of outliers. In the analyses of active time, one outlier (active time = 195, cook distance = 2.16) from an experimental nest was excluded. In the analyses of the frequency of aggression two experimental nests were outliers with respect to the frequency of aggression (one second chick, aggression = 17, cook distance = 20.51, and one first chick, aggression = 170, cook distance =48.09) and were therefore excluded from the analyses (Cook and Weisberg 1982, Crawley 2007).

In all models non significant terms (P-value > 0.05) were sequentially removed from the original model to obtain the minimal model (Crawley 2007). In one chick from the control group we could not obtain a blood sample large enough to enable us to determine androgen concentrations, hence this chick was not included in models containing androgen concentrations, nevertheless if androgen concentrations were not retained in the final model the data from this chick was incorporated in the new analysis.

RESULTS

First chicks

Androgen concentrations of first chicks (hatched from eggs laid before the experimental manipulation of male feet colour) did not differ between experimental and control groups (A4, controls: 280.79 ± 44.65 pg/ml, experimentals: 417.29 ± 107.29 pg/ml, Mann-Withney test, $U = 40$, $P = 0.31$; DHT, controls: 45.08 ± 8.74 pg/ml, experimentals: 41.05 ± 8.59 pg/ml, t-test, $t = 0.33$, $P = 0.74$; T, controls: 805.10 ± 70.88 pg/ml, experimentals: 898.45 ± 101.70 pg/ml, $t = -0.75$, $P = 0.46$). First chick behaviour did not differ between control and experimental broods; active time (General Linear Model, $F_{1,18} = 0.05$, $P = 0.82$), begging (Generalized Linear Model, $F_{1,19} = 0.16$, $P = 0.69$), frequency of aggressions (Generalized

Linear Model, $\chi^2_{1,18} = 1.64$, $P = 0.20$). However, first chick aggression was positively related to second chick begging behaviour (Generalized Linear Model, $\chi^2_{1,18} = 4.51$, $P = 0.03$). First chicks from experimental and control broods did not differ in their mass at hatching ($F_{1,18} = 0.60$, $P = 0.44$), body mass at 15d ($F_{1,18} = 0.61$, $P = 0.44$) or growth rate during the first 15d ($F_{1,18} = 0.03$, $P = 0.85$).

Effect of male feet colour manipulation on the mass, growth and behaviour of second chicks

Mass and Growth rate

Mass at hatching of second chicks was unaffected by the experimental manipulation of male feet colour (controls: 39.36 ± 1.20g, experimentals: 39.44 ± 1.30g; in all models, treatment, $F_{1,15} < 0.59$, $P > 0.53$; treatment * first chick hatching mass, P > 0.47) and no effect of chick sex (in all models, $F_{1,17} < 1.94$, $P > 0.18$), A4 ($F_{1,16} = 0.20$, $P = 0.67$), DHT ($F_{1,17} = 1.55$, $P = 0.22$) or T ($F_{1,17} = 0.94$, $P = 0.34$) was detected. The mass at hatching of second chicks was positively related with the mass at hatching of the first chick in the brood ($F_{1,19} = 20.07$, $P < 0.001$).

Growth rate of second chicks was unaffected by the experimental manipulation of male feet colour at 15 d (in all models, treatment, $F_{1,14} < 0.44$, $P > 0.51$; treatment * first chick growth rate, P > 0.29). We did not detect any effect of chick sex (in all models, $F_{1,16} < 0.88$, $P > 0.36$), A4 ($F_{1,15} = 0.03$, $P = 0.86$), DHT ($F_{1,16} = 1.28$, $P = 0.37$) or T ($F_{1,14} = 0.006$, $P = 0.93$). Second chick growth rate was not related to first chick growth rate ($F_{1,17} = 2.39$, $P = 0.14$).

Active time

The time that second chicks were active did not differ between groups (treatment $F_{1,19} = 0.67$, $P = 0.42$, all interactions with treatment $P > 0.20$). We did not detect any significant effect of chick sex (in all models $F_{1,18} < 0.16$, $P > 0.69$, all interactions with chick sex $P > 0.26$), or their plasma androgen concentration (A4 concentration $F_{1,18} = 2.16$, $P = 0.16$, all interactions with A4 $P > 0.51$; T concentration $F_{1,18} = 0.73$, $P = 0.40$, all interactions with T, $P > 0.71$; DHT concentration $F_{1,18} = 0.25$, $P = 0.62$, all interactions with DHT $P > 0.69$). The time that second chicks were active was not related to the active time of the first chick in the brood (in all models $F_{1,18} < 1.03$, $P > 0.32$, treatment*active time of the first chick $P > 0.20$) or the change in second chick body mass during the observation (in all models $F_{1,18} < 0.41$, $P > 0.53$).

Begging

Experimental second chicks spent less time begging than control chicks did, when the time spent begging by the first chick in the brood was included in the model (treatment $F_{1,17} = 4.38$, $P = 0.051$; begging by the first chick $F_{1,17} = 1.22$, $P = 0.284$; treatment* begging by the first chick $F_{1,17} = 9.34$, $P = 0.007$; Fig 1). Begging by second chicks was positively related to their change in body mass during the observation ($F_{1,17} = 4.81$, $P = 0.042$). We did not detect any effect of chick sex ($F_{1,17} = 0.02$, $P = 0.89$, all interactions with sex $P > 0.12$), A4 ($F_{1,16} = 0.001$, $P = 0.99$, all interactions with A4 $P > 0.33$), T ($F_{1,16} = 0.14$, $P = 0.71$, all interactions with T $P > 0.08$) or DHT concentrations ($F_{1,16} = 0.55$, $P = 0.48$, all interactions with DHT $P > 0.10$).

Aggression

Experimental second chicks increased their aggression when first chicks were more aggressive whereas second control chicks decreased it when the first chick's aggression was

included in the model (treatment $\chi^2_{1,16} = 9.45$, $P = 0.002$, first chick aggression $\chi^2_{1,16} = 23.03$, $P < 0.001$, treatment*first chick aggression $\chi^2_{1,16} = 15.71$, $P < 0.001$; Fig 2). Female chicks were 4 times more aggressive than males ($\chi^2_{1,16} = 26.35$, $P < 0.001$, all interactions with sex $P > 0.09$; Fig 3). The change on chick body mass during the observation was positively related with the aggression of the second chick ($\chi^2_{1,16} = 13.80$, $P < 0.001$), and A4 had a positive effect on the second chick aggression ($\chi^2_{1,16} = 6.16$, $P = 0.013$, Fig 4; all interactions with A4 $P > 0.16$). We did not detect any effect of T ($\chi^2_{1,16} = 0.001$, $P = 0.98$, all interactions with T $P > 0.23$) or DHT concentrations ($\chi^2_{1,16} = 2.42$, $P = 0.11$, all interactions with DHT $P > 0.15$) on the second chick's aggression.

DISCUSSION

This study suggests that female investment in eggs according to male feet colour during laying affects competitive abilities such as begging behaviour and aggression in blue footed booby chicks (Schwabl 1997, Eising and Groothuis 2003). Chicks fathered by experimental duller blue footed males spent less time begging and were more aggressive than control chicks; however, no differences in active time, mass at hatching and growth rate were detected. Furthermore, the frequency of aggressions by second chicks was positively related to their A4 plasma concentration but not to the concentrations of T or DHT, as previously described in gull chicks (Groothuis and Ros 2005).

Second chicks of experimental and control broods did not differ in their mass at hatching or growth rates during the first 15 days of life. Previous studies in birds have shown that high levels of circulating T and DHT reduce chick growth rates (*Gallus gallus domesticus*, Fennell and Scanes 1992; *Larus ridibundus*, Groothuis and Ros 2005; *Falco tinnunculus*, Fargallo et al. 2007). Additionally, it has been proposed that T could mediate a

trade off between chicks begging behaviour and growth rate by stimulating the costly expression of food solicitation and decreasing chick growth rate at the same time (Buchanan et al. 2007). In our study experimental chicks that have lower DHT concentration did not grow faster than control chicks. Furthermore, our results did not support the existence of a trade-off between begging behaviour and growth rate mediated directly by T, as no effect of androgens were detected on chick growth rate or begging behaviour. Nevertheless, we have to consider that our study only took in account nests in which both chicks survive until second chicks were 15d. First, this period may be too short to detect differences in growth rate and secondly the analysis may have included only nests with high quality parents (able to feed and rear two chicks) which could be a confounding effect.

The experimental modification of male feet colour during laying did not affect the time that second chicks were active but did influence the proportion of time spent in different activities. Chicks fathered by a male with experimentally dull blue feet during laying spent relatively less time begging than control chicks, when the time the first chick in the brood spent begging was considered. Begging behaviour in the blue footed booby seems to be an honest signal reflecting both needs and condition of chicks, since these beg more intensely when they are in poor body condition and when suffering recent food deprivation (Villaseñor and Drummond 2007). Hence, if at laying females are anticipating poor future rearing conditions using male feet colour as an indicator of paternal future contribution (Velando et al. 2005), females may adaptively produce chicks with lower energetic requirements. Blue footed booby females decrease yolk A4 concentrations when paired with an experimental male with dulled feet during an El Niño year with poor environmental conditions (Dentressangle et al. 2008), and 7-d old second chicks fathered by experimental males in apparent poor condition had significantly lower plasma A4 and DHT concentrations than control chicks, reflecting in the case of A4 a lower yolk A4 investment (Dentressangle and

Torres, in prep). It has been shown that high yolk androgen concentration increases chick metabolic rate (Tobler et al. 2007); hence, a decrease in yolk or chick plasma androgen concentrations may reduce the chick's metabolic rate and energetic requirement and thereby could favour the survival of chicks under poor rearing conditions. Furthermore, it has been suggested that A4 could be involved in the control of begging behaviour directly or through its modulating action on leptin levels (Clark 2002). As part of a neuroendocrine adaptation to fasting (Ahima et al., 1996), leptin regulates appetite and energy expenditure (Caro et al., 1996) and modulates the secretion of the growth hormone (Carro et al., 2007). High levels of leptin, through its actions on receptors located in the hypothalamus, provoke a reduction in food intake (Denbow et al., 2000) and *in vitro* experiments have shown that adipose tissue inhibits its leptin secretion when incubated in the presence of A4 (Piñero et al., 1999). Hence, a reduction of A4 concentration could increase leptin production and / or decrease the basal metabolic rate, which in turn could reduce begging behaviour. In the blue-footed booby, begging behaviour of second chicks elicit aggression from the first chick in the brood (Drummond et al. 1986). Thus, when expected paternal contribution is poor, indicated by dull blue feet, females may reduce the costs of aggressions between brood mates by producing second chicks that are less demanding.

Contrary to our prediction no relationship between circulating T or DHT and aggression of young chicks was found (Groothuis and Ros, 2005). In agreement with previous studies on blue-footed booby chicks, T did not seem to be involved in the control of chick aggression (Nuñez de la Mora et al. 1996, Ramos-Fernandez et al. 2000). Nevertheless, we detected a small and positive correlation between A4 and chick aggression. A positive effect of A4 on aggressive behaviour has been reported in mammals (Rats, *Rattus norvegicus*, Christie and Barfield 1979; Human youngsters, Azurmendi et al., 2006; Ramirez, 2003; Spotted hyenas, *Crocuta crocuta,* Glickman et al., 1987, Dloniak et al., 2006, lemurs, *Lemur*

catta, Drea et al., 2007) and Gold fish (*Carassius auratus,* Poling et al., 2001). In rats, the expression of aggressive behaviour is known to be influenced by the positive effects of A4-activated vasopressin on the projections of the bed nucleus of the stria terminalis and the centromedial amygdale in the brain (Ferris, 1992, Villalba et al., 1999). To our knowledge, this is the first study to report a positive correlation of A4 on aggressive behaviour in chicks and supports the idea that this androgen could be involved in the production of more competitive phenotypes in social groups (Cariello et al. 2006, Gil et al. 2007, Dentressangle, in prep).

Interestingly, second experimental chicks (that have lower concentration of A4 and DHT than control chicks, Dentressangle and Torres, in prep) were more aggressive than control chicks. This result suggests that the control of chick aggressive behaviour may depend not only on androgens but also in other factors such as corticosterone concentrations (Groothuis and Ros, 2005). In the blue footed booby, subordinate chicks of 15-20 days have higher concentration of corticosterone than dominant or singleton chicks (Nuñez de la Mora et al. 1996) which seems to facilitate submissive postures (Vallarino et al. 2006), whereas in dominant chicks this same hormone seem to increase aggressiveness (Ramos Fernandez et al. 2000). It is therefore possible that complex interactions between A4 and corticosterone are responsible for this observed trend. Moreover, even if the sex ratio did not differ significantly between groups, our experimental second chick group had more females (64%) than the control group (40%, Dentressangle and Torres, in prep), and females were 4 times more aggressive than males. Thus, the female biased sex ratio in the experimental group could also contribute to the greater levels of aggression by second chicks observed in these broods.

The higher level of aggression observed in female chicks was not due to differences in plasma androgen concentrations between sexes; at 7d male and female chicks did not differ in their hormonal profile (Dentressangle and Torres, in prep). It has been suggested that sons and

daughters might differ in competitive ability (aggressiveness) for food delivered by the parents and under adverse feeding conditions, the more aggressive sex (generally the larger sex) might out-compete the less aggressive sex, leading to higher nestling mortality in the latter (Hasselquist and Kempenaers 2002). However, in the blue footed booby adult females are 31% heavier than males, and female chicks seem to suffer higher risk of mortality than males under poor rearing conditions (Torres and Drummond 1997, Velando and Alonzo-Alvarez 2003). As female chicks have to deal with a higher probability of death, it is therefore possible that natural selection has favoured the evolution of more aggressive females during the chick rearing period to increase their survival probability, as this occurs in second chicks of species with obligate brood reduction (Drummond et al. 2003). Under good rearing conditions, the sexual composition of the brood did not influence the survival or growth of the first or second chick until fledging (Drummond et al. 1991), and no long term effects on female survival and reproduction according to her status (dominant/subordinate) during early life have been detected (Drummond et al. 2003, Carmona, 2008). However, when rearing conditions are poor more aggression can lead to a more efficient brood reduction (Drummond & Garcia-Chavelas 1989).

To conclude, this study supports the hypothesis that differential allocation in eggs according to male attractiveness can influence chicks begging and aggressive behaviour (Schwabl 1993, Gil et al. 1999, Groothuis et al. 2005). Previous studies on the blue footed booby suggest that when male feet colour deteriorates during egg laying, females may strategically favour brood reduction by laying smaller eggs with lower concentrations of A4 (Velando et al. 2006, Dentressangle et al. 2008). This idea was supported by a recent experiment that showed that second eggs fathered by a male with duller feet colour have lower hatching success and their chicks have lower cellular immune response (Dentressangle and Torres, in prep). In the present study we could not evaluate whether female allocation in

eggs facilitated brood reduction, as the expected poor rearing conditions due to a male in poor condition did not match the actual rearing conditions of the brood, and only broods where both chicks survived were used for the behavioural observations. However, begging behaviour and the frequency of aggressions performed by second chicks were influenced by female investment at the egg stage. Whether these changes in the behaviour of second chicks are adaptive remains to be tested. However, under good rearing conditions a decrease in the begging behaviour of second chicks may increase their survival probabilities by saving energy and decreasing first chicks' aggression towards the second chick, while under poor rearing conditions a decrease in begging behaviour and an increase in the frequency of aggressions may facilitate brood reduction. Our study suggests that blue-footed booby females are able through differential investment in the eggs to modify the phenotype (personality) of their chicks.

ACKNOWLEDGMENTS

We are grateful to M. Cerbón, A. Córdoba-Aguilar and H. Drummond, for helpful comments and discussion during the study, A. Gonzalez-Voyer for comments on the manuscript, to E. Villaseñor, J. Morales and G. Trejo Macías for their great help during field work, and to the Laboratory of steroid hormones of the "Instituto Nacional de Ciencias de la Salud y de Nutrición", especially to L. Boeck and R. Chavira for androgens determination. The project was supported by the Universidad Nacional Autónoma de México (UNAM, PAPIIT IN211406) and CONACYT (47599). Logistic support was provided by the Armada de México, the staff from the Parque Nacional Isla Isabel and the fisherman from San Blas, Nayarit. The experiments comply with the current laws of Mexico, permissions were granted by SEMARNAT and the Parque Nacional Isla Isabel. During the study F. Dentressangle was supported by a scholarship for graduate studies from UNAM.

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Figure 1. Proportion of active time begging by second chicks in control (n=10) and experimental (n= 11) broods according to the proportion of active time spent begging by the first chick in the brood.

Figure 2. Frequency of aggressions emitted by second control (n=10) and experimental (n=8) chicks in relation to the number of aggressions emitted by the first chick in the brood.

Figure 3. Mean number of aggressions (± s.e.) emitted by male (n=9) and female (n=9) second chicks.

Figure 4. Effect of plasma A4 concentration (pg/ml) of 7d old chicks (control ● and experimental ○) on the aggressive behaviour of second hatched chicks at the age of 10 days. The residuals of the model including treatment, chick sex and change in body mass during the observation are shown.

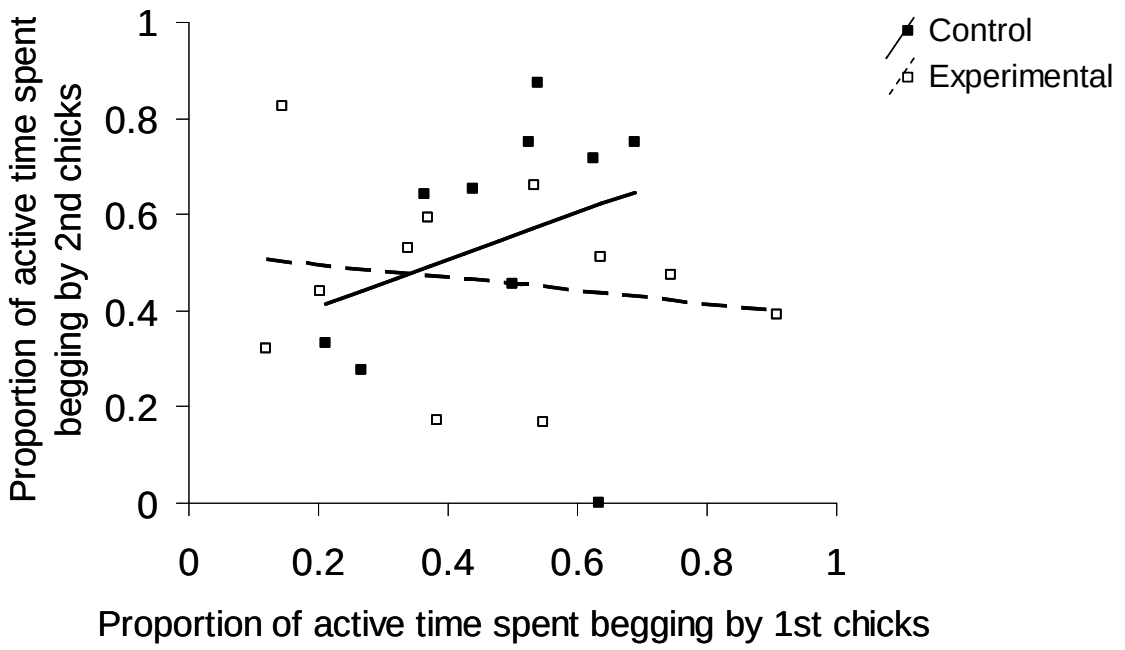
Proportion of active time spent begging by 2nd chicks
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Proportion of active time spent begging by 1st chicks
Control
Experimental

Figure 1

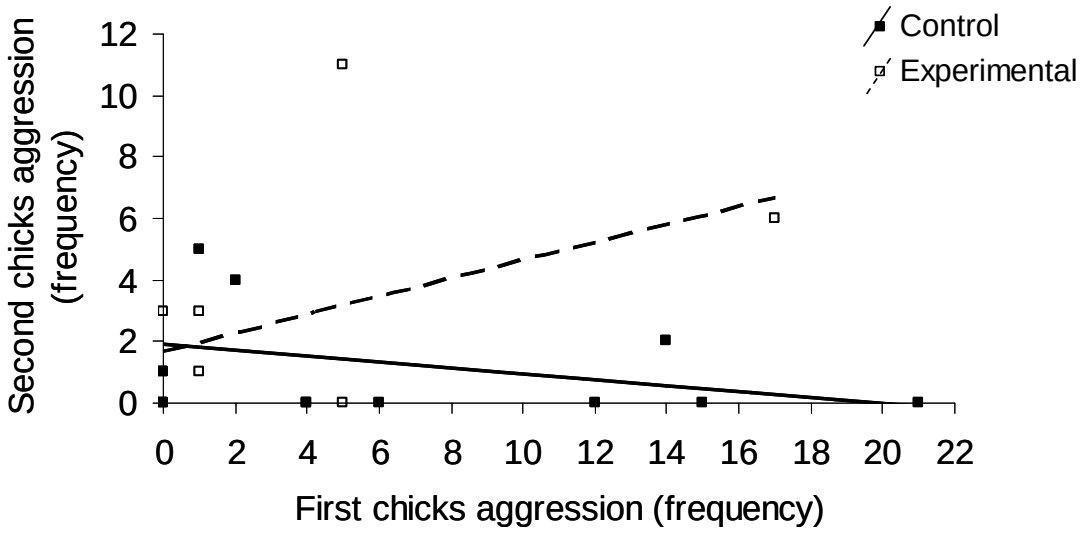
Second chicks aggression (frequency)
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
22
First chicks aggression (frequency)
Control
Experimental

Figure 2

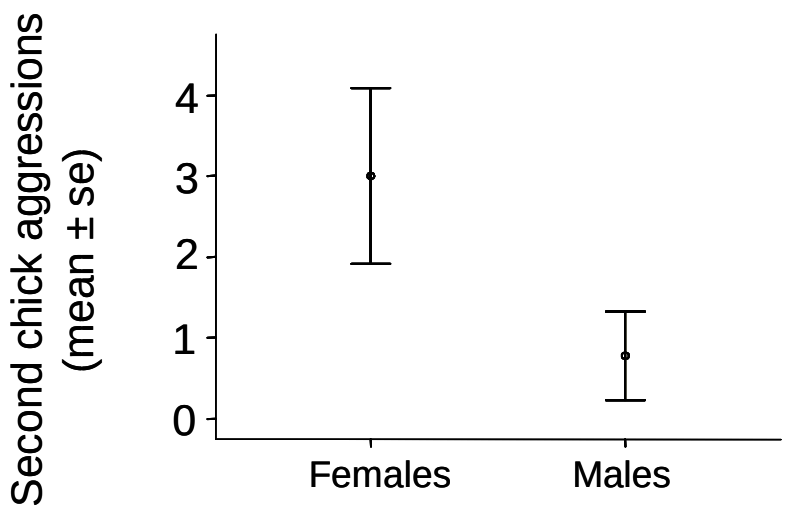

Figure 3

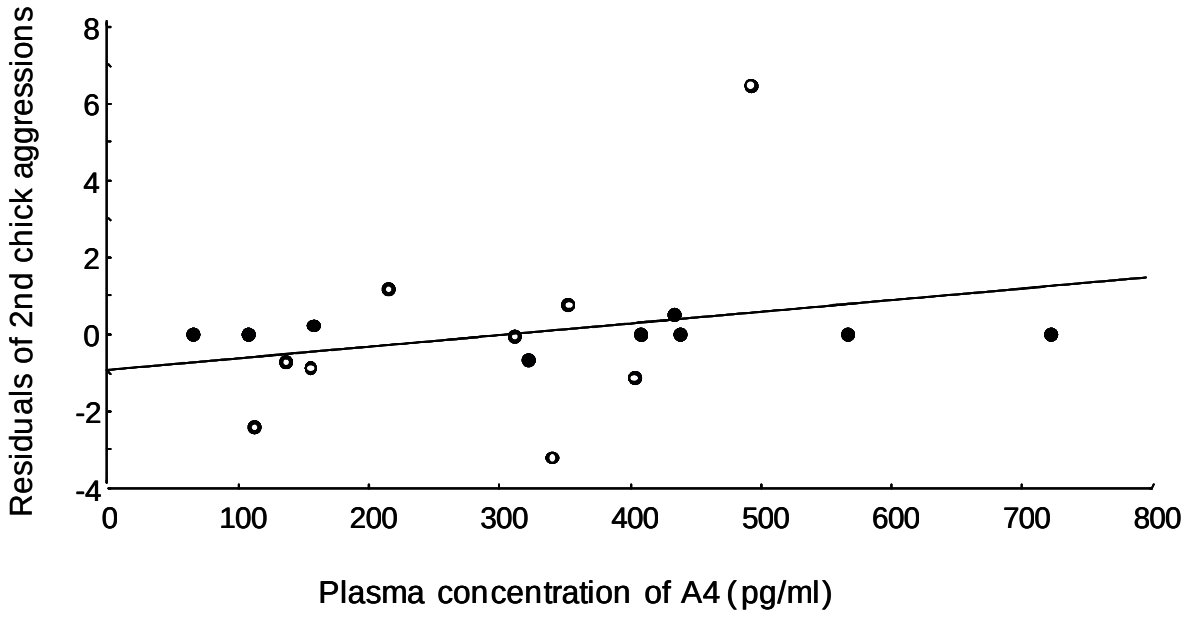
Residuals of 2nd chick aggressions
8
6
4
2
0
-2
-4
0
100
200
300
400
500
600
700
800
Plasma concentration of A4 (pg/ml)

Figure 4

Conclusiones y perspectivas

Los efectos maternos se reconocen como un mecanismo adaptativo por el cual las condiciones experimentadas por las hembras a lo largo de su vida se transmiten a su progenie, preparándola para enfrentar un cierto tipo de ambiente (Mousseau y Fox 1998). Dependiendo de las condiciones presentes (por ejemplo, el valor del evento reproductivo, la disponibilidad de alimento, la calidad de su pareja, la densidad de anidación; Mousseaux y Fox 1998; Groothuis et al. 2005; Gil 2008) y pasadas (por ejemplo, el estrés durante las etapas tempranas del desarrollo; Gil et al. 2004), las hembras ajustan su inversión en la reproducción para maximizar su propia adecuación (Marshall y Uller 2007). De acuerdo con lo anterior, se ha documentado que las hembras pueden modificar el tamaño de sus huevos y su composición bioquímica, influyendo así en la probabilidad de supervivencia de las crías (Cunningham y Russell 2000; Gil et al. 1999). Por ejemplo, en aves se ha reportado un efecto positivo del tamaño del huevo sobre la probabilidad de eclosión y el tamaño al nacer (Christians 2002), y las concentraciones de andrógenos en el vitelo de los huevos pueden acelerar el desarrollo embrionario, incrementar la tasa de solicitud de alimento de las crías, estimular el crecimiento post eclosión y afectar el estatus social durante la etapa adulta (Eising et al. 2001; Eising y Groothuis 2003; Schwabl 1997). Un tipo particular de efectos maternos ocurre cuando el atractivo de la pareja refleja su calidad y podría ser un indicador del futuro cuidado parental (hipótesis de inversión diferencial: Burley 1986, 1988).

El bobo de patas azules, *Sula nebouxii*, es un modelo apropiado para probar si las hembras ajustan su inversión en la reproducción de acuerdo a las condiciones ambientales y al atractivo de su pareja. El bobo de patas azules es un ave marina que

anida en la isla Isabel (en el pacifico de las costas de Nayarit) y está sujeta a una variación interanual importante en la disponibilidad de alimento debido a la presencia del fenómeno climático "El Niño". En esta especie, el color de las patas de los machos es una característica bajo selección sexual (Torres y Velando 2003) que se relaciona positivamente con la condición nutricional, la capacidad inmune de los machos (Velando et al. 2006) y probablemente con la inversión paterna durante la crianza (Velando et al. 2005).

En este estudio primero evaluamos si las hembras del bobo de patas azules modifican su inversión en los huevos (tamaño y concentración de andrógenos) de acuerdo a las condiciones ambientales y al color de las patas de la pareja (hipótesis de la inversión diferencial; Burley 1986, 1988). En segundo término, evaluamos si la inversión diferencial en los huevos afecta la probabilidad de eclosión, la respuesta inmune (capítulo 2) y tasa de crecimiento de las crías, así como su personalidad (conducta de solicitud de alimento y agresión, capítulo 3).

Principales resultados

Las hembras del bobo de patas azules ajustaron su inversión en la puesta en función de las condiciones ambientales y del atractivo de su pareja. Comparado a un año con buenas condiciones para la crianza, durante un año de El Niño, con una disponibilidad de alimento limitada, las hembras pusieron un segundo huevo mas grande que el primero (volumen y peso), y como se ha reportado en otras especies, con mayor concentración de androstenediona (A4) (Verboven et al. 2003; Gasparini et al. 2007). Se ha sugerido que las hembras con una baja condición corporal debido a la escasez de alimento podrían asignar mas andrógenos al vitelo para compensar los posibles efectos negativos debidos a las pobres condiciones de crianza y así favorecer el desarrollo y

supervivencia de sus crías (Verboven et al. 2003; Gasparini et al. 2007). Los resultados de nuestro estudio apoyan la hipótesis de que las hembras están ajustando su inversión de acuerdo a las condiciones ambientales, anticipando las futuras condiciones de crianza e invirtiendo de una manera que maximiza su adecuación (Mousseau y Fox 1998; Trivers 1972; Stearns 1992; Sheldon 2000). Al aumentar el tamaño y las concentraciones relativas de A4 en el segundo huevo cuando las condiciones ambientales son pobres, las hembras podrían estar favoreciendo las probabilidades de sobrevivencia de la segunda cría al disminuir las asimetrías en tamaño a la eclosión entre crías de una misma nidada, y mejorando la capacidad inmune y habilidades competitivas de las segundas crías (Verboven et al. 2003; Gasparini et al. 2007; Capítulos 2 y 3). Sin embargo, cuando el color de patas del macho se modifico a un azul mas oscuro, simulando un macho en baja condición (Torres y Velando 2003; Velando et al. 2006) y probablemente un nivel de inversión paterna bajo durante la crianza (Velando et al. 2005), las ventajas que parecía dar la hembra al segundo huevo durante un año de El Niño desapareció. Las hembras redujeron el tamaño del segundo huevo (masa y volumen), atrasaron la puesta del segundo huevo, y redujeron las concentraciones relativas de A4 en el vitelo (capítulo 1). Estos resultados apoyan la hipótesis de que las hembras ajustan su inversión en los huevos de acuerdo al atractivo de su pareja (Burley 1986 y 1988) y que podrían estar favoreciendo la reducción de la nidada cuando las expectativas de éxito de la nidada son bajas (Velando et al. 2006), es decir cuando la aparente condición de la pareja es baja y las condiciones ambientales para la reproducción son pobres.

De acuerdo con la hipótesis de Velando et al. (2006), los segundos huevos que tuvieron un padre cuyo color de patas se modificó a un azul oscuro tuvieron un éxito de eclosión menor a los huevos controles, aún cuando las hembras en este experimento no

redujeron el tamaño del segundo huevo (Capitulo 2). Aun más, los huevos en el grupo experimental fueron fertilizados en una menor proporción que los huevos controles, indicando un posible control de las hembras sobre la fertilización de los huevos. Se ha reportado que las hembras de bobo de patas azules disminuyen sus tasas de cortejo y copula cuando se modifica experimentalmente el color de la patas de su pareja hacia un azul mas oscuro (Torres y Velando 2003); este ajuste conductual por parte de las hembras podría explicar la menor proporción de huevos fertilizados en el grupo experimental que en el grupo control (Capítulo 2). Sin embargo, no se pueden excluir otros posibles mecanismos crípticos de elección de la hembra como la posibilidad de que las hembras expulsen esperma cuando el atractivo de su pareja se ve deteriorado, como ha sido documentado en otras especies (Birkhead y Moller 1992; Adkins-Regan 1995; Pizzari y Birkhead 2000; Persaud y Galef 2005).

La inversión diferencial en los huevos por parte de las hembras de acuerdo al color de las patas de su pareja no solamente afectó la probabilidad de eclosión sino también estuvo relacionada con la respuesta inmune celular y con algunas variables conductuales de las crías. Respecto a las crías del grupo control, los del grupo experimental (nacidos de huevos puestos por las hembras apareadas con los machos menos atractivos) tuvieron una concentración menor de A4 y DHT a los 7 días de edad, así como una menor respuesta inmune celular a los 15 días de edad (capitulo 2). En cuanto a las variables conductuales, las crías experimentales pasaron relativamente menos tiempo solicitando y fueron mas agresivas que las crías controles (Capítulo 3). Sin embargo, la tasa de crecimiento de las crías no se vio afectado por la manipulación de color de las patas de los machos, lo que sugiere que una disminución del nivel de A4 en los huevos podría reducir la tasa metabólica de las crías como ocurre en el pinzón cebra cuando disminuyen los niveles de T en el huevo (Tobler 2007).

Globalmente, podemos concluir que, al momento de poner los huevos, las hembras del bobo de patas azules ajustan su inversión de acuerdo a varios factores susceptibles de reflejar las futuras condiciones de crianza, como la calidad del ambiente (Mousseau y Fox 1998; Verboven et al. 2003, Gasparini et al. 2007) y el atractivo de su pareja (Burley 1986, 1988; Gil et al. 1999; Tanvez et al. 2004; Loyeau et al. 2007). Nuestros resultados resaltan la importancia de los efectos maternos sobre la adecuación de las crías y refuerzan la idea de que los efectos maternos deben de ser tomados en cuenta en los estudios de selección sexual. La inversión diferencial de la hembra de acuerdo al ambiente y al atractivo de su pareja puede afectar la probabilidad de supervivencia de la progenie, la respuesta inmune o la conducta, y por lo tanto puede ser una fuente importante de variación en la calidad de las crías (Price 1998).

Adicionalmente, los resultados del segundo y del tercer capitulo apoyan fuertemente la idea de que la calidad del huevo no esta únicamente relacionada con su tamaño (Christian 2002; D'Alba y Torres 2007), sino también en gran parte con su composición bioquímica (Nager et al. 2000, Clifford y Anderson 2002). La inversión diferencial de andrógenos (A4) en los huevos influyo sobre la respuesta inmune y las habilidades competitivas de las crías de bobo de patas azules, como ha sido reportado en otras especies (Müller et al. 2005; Navara et al. 2005, 2006; Schwabl 1993, 1997; Eising et al. 2001; Groothuis y Ros 2005). Sin embargo, y de acuerdo con estudios previos realizados en el bobo de patas azules no detectamos efectos de la testosterona sobre la agresión de las crías (Nuñez de la Mora et al. 1996; Ramos-Fernandez et al. 2000). Tampoco observamos un efecto de dicho andrógeno sobre la respuesta inmune o la tasa de solicitud de alimento a diferencia de lo reportado en otras especies (Navara et al. 2005; Müller et al. 2005; Schwabl 1993; Eising y Groothuis 2003; Groothuis y Ros 2005). La filogenia parece ser un factor importante para los diferentes efectos

observados con los andrógenos (Carielo et al. 2006; Gil et al. 2007). Los altos niveles de A4 son característicos de las especies que no son paseriformes y podrían en parte explicar porque no detectamos efectos de la T sobre las variables que analizamos (Carielo et al. 2006; Gil et al. 2007). Adicionalmente, la latitud es otro parámetro muy importante en los niveles de T en plasma; los machos de especies tropicales presentan una menor variación en sus niveles de T comparados con especies de zonas templadas (Goymann et al. 2004), lo que podría explicar, al menos en parte, la falta de relación entre la T y las variables analizadas en este estudio. Nuestros resultados sugieren que se deben de analizar de forma independiente los efectos de los diversos andrógenos ya que pueden tener efectos diferentes según la especie considerada (Gil et al. 2007; Gil 2008). El bobo de patas azules mostró concentraciones de A4 en el vitelo mucho mayores a las de T (aproximadamente 20 veces mas altas), lo que coincide con los resultados obtenidos de un estudio comparativo en el cual las altas concentraciones de A4 en el vitelo fueron asociadas con aves coloniales pesadas (Gil et al. 2007).

La A4 que durante mucho tiempo ha sido considerada como una hormona "débil", parece estimular la respuesta inmune (Yao y Shang 2005; Gil et al. 2006) y la agresión en las crías de bobos de patas azules (Capítulos 2 y 3). La relación positiva encontrada en nuestro estudio entre el nivel de agresión de las crías y la A4 ha sido reportada en mamíferos y peces (ratas, *Rattus norvegicus*, Christie and Barfield 1979; hyenas, *Crocuta crocuta,* Glickman et al. 1987; lémures, *Lemur catta,* Drea et al. 2007; pez amarillo, *Carassius auratus*, Poling et al. 2001; humanos, Azurmendi et al. 2006; Ramirez 2003), pero este estudio es el primero que reporta tal efecto en aves. Este resultado es muy novedoso y sugiere que la A4 podría estar involucrada en la producción de fenotipos competitivos en grupos sociales (Cariello et al. 2006; Gil et al. 2007). Adicionalmente, Clark (2002) sugirió que la A4 podría estar involucrada en el

control de la solicitud de alimento, de manera directa (como se ha reportado para la T; Gil 2003; Groothuis et al. 2005), o indirecta a través de un control sobre la leptina. Los resultados del Capítulo 3 sugieren que la A4 podría tener efectos sobre la solicitud de alimento en el bobo de patas azules, ya que las crías experimentales solicitaron menos que las crías controles y tienen concentraciones menores de A4 que estas últimas. Sin embargo, este efecto no parece ser directo, ya que no encontramos relación entre esta hormona y el tiempo que la cría pasó solicitando. Futuros estudios deberían investigar la posible interacción entre los niveles de leptina y A4 sobre la solicitud de alimento.

Así, en el bobo de patas azules la inversión materna esta influida por el color de las patas del macho, lo que sugiere que los efectos maternos en esta especie podrían tener implicaciones en la evolución y mantenimiento de este ornamento (Kirkpatrick y Lande 1989; Strasser y Schwabl 2004; Eising et al. 2006). Si las hembras invierten más en un evento reproductivo cuando están apareadas con individuos más atractivos, las crías de padres con color de patas azul turquesa, presumiblemente padres que invierten más en la crianza, tendrían más recursos. Las condiciones que las crías experimentan durante el desarrollo pueden tener efectos a largo plazo en la expresión de características sexuales; crías que crecen en mejores condiciones despliegan mejores ornamentos cuando llegan a la etapa de adulto. En particular, la modificación de las concentraciones de andrógenos en el vitelo de los huevos ha sido identificada como un parámetro relevante en la expresión de ornamentos en la etapa adulta. Altos niveles de andrógenos en el vitelo parecen acelerar la expresión de los ornamentos (Eising et al. 2006) y están asociados positivamente con el tamaño del ornamento así como con conductas de cortejo y de defensa de territorio (Strasser y Schwabl 2004; Eising et al. 2006; Panzica et al. 2005). Por lo tanto, si las hembras aumentan su inversión en las crías cuando están apareadas con machos atractivos, estas crías serán a su vez más

atractivas y tendrán mayores posibilidades de reproducirse y dejar descendencia. Consecuentemente, la asignación diferencial en función del atractivo de la pareja podría acelerar la tasa a la que evoluciona la característica bajo selección sexual (Kirkpatrick y Lande 1989).

En un gran número de especies en las cuales opera la elección de pareja, los ornamentos durante el cortejo juegan un papel fundamental en las decisiones reproductivas de las hembras. Sin embargo, este estudio resalta la importancia de los ornamentos aún después del establecimiento de la pareja. En el bobo de patas azules la evaluación del macho a través de una señal dinámica como el color de las patas, continúa después del periodo de cortejo, durante la puesta. A diferencia de una señal estática, como el color del plumaje, una señal dinámica, como el color de patas, puede reflejar la condición actual de un individuo, y permitir a su pareja evaluarlo de manera continua y ajustar el esfuerzo parental según las condiciones esperadas para la crianza. La habilidad de las hembras para modificar su inversión en la puesta de acuerdo al color de las patas del macho podría ser una fuerza de selección que favorezca en los machos el mantenimiento de un color de patas atractivo durante el cortejo y la puesta. Debido a que el color azul turquesa de las membranas de las patas que despliegan los machos durante el cortejo es el resultado del efecto de la estructura de colágeno y pigmentos carotenoides (Velando et al. 2006; Morales et al. 2009), y los carotenoides son recursos limitantes, es posible que mantener un color de patas atractivo sea costoso y solo los machos de buena calidad o en buena condición puedan mantener esta señal y asegurar así una mayor inversión por parte de las hembras. Sería interesante en estudios posteriores investigar si esta evaluación de la pareja mediante el color de las patas se extiende hasta el periodo de crianza, permitiendo a los adultos ajustar su esfuerzo de forrajeo y alimentación de las crías.

En la última década los estudios sobre los efectos maternos a través de la inversión diferencial se han multiplicado. Sin embargo, faltan aún por resolver muchas preguntas a nivel de los mecanismos fisiológicos (e.g. ¿cómo pasan los andrógenos a los huevos? ¿se trata de un mecanismo pasivo o activo?, ¿cuáles son los genes que son activados o apagados durante el desarrollo debido a la presencia de andrógenos?, ¿cuáles son los efectos organizacionales y "activacionales" de los andrógenos?), así como estudios en los que se evalúen las consecuencias de la inversión diferencial en la adecuación de los padres y de las crías. En el bobo de patas azules los efectos maternos a través de la inversión diferencial en los huevos parecen ser un poderoso mecanismo que puede influir en la calidad, habilidad competitiva y sobrevivencia de las crías.

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IS ANDROSTENEDIONE INVOLVED IN THE PRODUCTION OF COMPETITIVE PHENOTYPES?

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During the embryogenesis, steroid hormones in birds are known to regulate many physiological functions such as the metabolism of carbohydrate, fat and protein or the regulation of water and mineral balance that are critical for the development of an organism and its post embryonic survival (Jenkins and Porter, 2004; Lea et al., 1998; Piñero et al., 1999). Because hormonal responses occur only when appropriate receptors are available, the fact that specific steroid receptors are located in the central nervous system across the entire vertebrate class, suggests that these hormones have evolutionary stable roles not only during the differentiation and postnatal development of target cerebral circuits, but also in the maintenance of their functions in the adult brain (Panzica et al., 1996; Schlinger, 1998; Jenkins and Porter, 2004). Different studies focused on the effects of gonadal steroids on the sexual differentiation of brain during embryogenesis and on the expression of reproductive behavior in the adulthood found that gonadal steroids may act on the neuronal web having organizational effects as well as activational effects (Adkins, 1979; Adkins-Regan, 2007; Schlinger, 1998; Schlinger et al., 2001; Panzica et al., 2005). For example, during the sexual differentiation of the brain, the organizational effects result in anatomically different structures which "establish the foundation for the performance of masculine or feminine behavior by adults" (Adkins, 1979; Adkins-Regan, 2007; Panzica et al., 2005). In contrast, the activational effects are generally transient and result in the expression of courtship or singing

behaviours during the reproductive season in adults (Schlinger et al., 2001; Adkins-Regan, 2007).

In addition to the endogenous production of gonadal steroids, another source of androgens for developing birds comes from the egg yolk via maternal effects. Recently a number of studies have shown that mothers can influence offspring quality and the rate of embryonic development through to embryo exposure to maternal steroids such as testosterone (T), dihydrotestosterone (DHT) and androstenedione (A4). Maternally derived androgens are present in the yolk of avian eggs (Schwabl, 1993; for review see Groothuis et al., 2005) and have multiple effects on morphology, physiology and behavioural components that can strongly influence the chick's probability of survival, and even shape the phenotype of the adult bird. So far research has focused in how offspring characteristics are affected by yolk hormones and knowledge about the metabolic route followed by androgens in the embryo is poor. However, it has been found that at very early stages in development functional androgenic receptors exist in the hindbrain prior to the endogenous synthesis of steroids in the gonads (Godsave et al., 2002), suggesting that maternal yolk androgens have the potential to influence the embryo development. For instance, high yolk T concentrations stimulate the growth of the *musculus complexus* (the muscle involved in breaking the shell at hatching and in neck movements during begging), which in turn may affect the chick´s begging behaviour latter (Lipar and Ketterson, 2000). High levels of androgens also modify the length of the embryonic period (Sockman y Schwabl 2000, Eising et al., 2001, Eising and Groothuis, 2003, von Engelhardt et al. 2005), stimulate chick growth (Schwabl 1996, Eising et al., 2001 Groothuis et al 2005; Navara et al., 2006, Pilz et al 224, von Engelhardt et al. 2006, Muller et al 2007, but see Sockman y Schwabl 2000) and increase a chicks' active time and begging behaviour (Schwabl 1996, Eising and Groothuis, 2003, von Engelhardt et al. 2005, Boncoraglio et al 2006). Studies that have followed birds until adulthood have revealed long

term effects of yolk androgens: when compared to birds hatched from eggs with lower levels of T, adults hatched from eggs with higher levels of T obtained higher social status (Schwabl, 1993), exhibited larger sexual secondary traits and obtained and defended their resources more efficiently (Strasser and Schwabl, 2004, Eising et al. 2006). Despite these positive effects, some studies have identified that elevated T levels in yolk can also negatively affect hatching success (Navara et al., 2005), reduce the immune response (Müller et al., 2005; Groothuis et al 2005, Navara et al., 2005), increase the basal metabolic rate (Tobler et al., 2007), generate biased sex ratios (Rubolini et al., 2006), and even compromise chick survival (Sockman and Schwabl, 2000).

It has been shown that the concentrations of yolk androgens vary within clutches according to laying order (Schwabl, 1993, 1997; Gil et al., 1999; Sockman and Schwabl, 2000; Reed and Vleck, 2001; Eising and Groothuis, 2003) and between clutches according to different parameters such as environmental and breeding conditions experienced by the mother (Groothuis and Schwabl, 2002; Whittingham and Schwabl, 2002; Mazuc et al., 2003; Gil et al., 2004a; Tschirren et al., 2004; Cariello et al., 2006; Dentressangle et al., 2008), the mating system (Gwinner and Schwabl, 2005) and the mate attractiveness (Gil et al., 1999, 2004b; Tanvez et al., 2004, Dentressangle et al. 2008). Schwabl (1997) suggested that the differential allocation of yolk androgens could be "a hormonal mechanism for parental favouritism" that can influence sibling competition through differences in body condition and behaviour among chicks. For example, a positive increase with laying order in yolk T can reduce the effects due to hatching asynchrony between chicks by making the last chick more competitive. Alternatively, a negative relationship between yolk T and laying order would enhance differences between sibs caused by asynchronic hatching and facilitate the elimination of the junior chick (Schwabl, 1997).

Despite the three main androgens present in avian eggs are T, DHT, and A4, there is only one published study in which the differential effect of each of these hormones has been addressed (Gil et al. 2007). Since the work of Schwabl (1993) behavioural ecologists have focused principally on studying the effects of yolk T and have paid limited attention to the effect of A4 probably due the fact that T (which is strongly correlated to DHT in passerine birds r=0.9; Schwabl et al 2007) is an important mediator in the regulation of behaviour in male vertebrates and its effects on morphology, behavior and physiology have been extensively studied. In addition, in terms of its androgenic effect, A4 is usually regarded as a weaker androgen, less potent than T or DHT (*in vitro* tests that measured affinity for the androgen receptor; Sonneveld 2006). However, it seems rather meaningless to judge the potential androgenic effect of the suite of possible yolk androgens without knowing the precise chemical matrix in which these androgens act in the embryo (Gil 2008). For example, a recent study with castrated hamsters (*Mesocricetus auratus*) suggests that A4 is much more effective than T in restoring male sexual behaviour, especially ejaculatory behaviour (Arteaga-Silva et al., 2008). After exogen steroid administration a concentration of 300 µg / day of T was necessary to obtain the same result produced by the administration of 50 µg / day of A4 (Arteaga-Silva et al., 2008). Since A4 can be converted into T by the enzymatic action of the 17 β hydroxystreroid deshydrogenase (17HSD; Horton and Tate, 1966), its effects will depend on the availability of this enzyme which has been found to be present and functional in developing avian embryos (Bruggeman et al. 2002).

Recently, both a comparative study in birds by Gil et al. (2007) and a new review of hormones in avian eggs (Gil 2008) highlighted the importance of considering each androgen individually since positive relationships between A4 and T are not very strong, and because some of the relationships between androgen concentration levels and ecological behaviour variables are only found for either T or A4 suggesting that these two androgens may have

different effects on the development of the embryo. For example, while high yolk A4 were associated with colonial life and heavier birds this association was not found for yolk T (Gil et al., 2007). On a similar line, Groothuis and Schwabl (2002) had suggested that in birds, embryos of large species would require higher levels of androgen during development than embryos of smaller species. These authors also speculated that the high levels of A4 in the embryo could have a protective function by counteracting the possible toxic effects of high T levels (Groothuis and Schwabl, 2002). Yolk A4 has also been shown to have positive effects on chicks' growth rate (Gil et al., 2006), and on the cellular immune response (Gil et al., 2006; Dentressangle and Torres, in prep). Hence, A4 may be involved in the development of competitive phenotype in a social environment (Carrielo et al., 2006; Gil et al. 2007).

Despite the attractiveness of this hypothesis, and the fact that some investigations, principally on mammals, indicate an influence of A4 on physiological and behavioural traits, the studies of the effects of yolk A4 on birds´ development and behavioural are scarce (Carrielo et al., 2006; Gil et al., 2007). To highlight the relevance of understanding the effects of A4 on the development and phenotype of bird embryos, in this paper I will review the current knowledge on the principal effects of A4 on physiological and behavioural characteristics, and evaluate whether the available data supports the hypothesis proposed by Gil and colleagues (2007) relative to the role of A4 in the development of competitive phenotypes. For the purpose of this review, a competitive phenotype would be a chick that, within the brood, will grow faster, beg more intensively, have better immune response and higher behavioural dominance rank than its siblings (Schwabl, 1997; Groothuis et al., 2005; Gil et al., 2007). I will review the potential effects of A4 on each of these traits.

GROWTH RATE

Anabolic effects of A4

In birds a large number of studies in a variety of species have found that nestlings hatching from androgen-injected eggs have shorter incubation periods than nestlings from control-injected eggs (review in Gil 2008), presumably because yolk androgens can accelerate embryonic growth rate due to their anabolic effects. Along with other steroids, A4 is widely used by athletes and bodybuilders to gain muscle mass and enhance their performance (Stevens, 1995). The anabolic effects of A4 on myogenesis have been observed in *in vitro* experiments. A4 up-regulates the MyoD protein, an essential transcription factor for myogenesis differentiation, and stimulates the expression of the myosin heavy chain in a dose- depend manner (Jasuja et al., 2005). These authors have also reported *in vivo* experiments with hypogonadal men that show a positive effect of A4 on muscle mass and muscle strength. It was unclear however, whether the effects were due to the direct action of A4 on myogenesis or to its conversion into T. Nevertheless, for its myogenic potential, A4 is classified as anabolic steroid under the controlled substance act of USA (1990) (Jasuja et al., 2005).

Due to the potential conversion of A4 into T, the direct effects of A4 are sometimes difficult to establish. Nevertheless, A4 has been shown to enhance the proliferation of various types of cells other than myocites (Baratta et al., 2000; Maliqueo et al., 2004; Yao and Shang, 2005): mammary epithelial cells in mice (Baratta et al., 2000) and cells of the endometrium in women (Maliqueo et al., 2004), two tissues that undergo anabolic transformation during reproduction. Maliqueo et al. (2004) observed reduced apoptosis when endometrial cells were incubated with A4, as well as up regulation in the expression of estrogenic receptors. Such potential effects of A4 to alter the normal pattern of cell apoptosis may lead to negative outcomes such as tumor development in women with polcystic ovarian syndrome (Maliqueo et al., 2004).

A4 is also involved in the maintenance of skeleton integrity as it has been evidenced in rats and humans (Dubridge et al., 1990; Marshall et al., 1977; Devogelaer et al., 1987). A4 regulates bone turn over (Lea et al., 1998) and reduced concentrations of this hormone are associated with high risk of bone fractures (Marshall et al., 1977). There is one study to date that shows a stimulating effect of yolk A4 on chick growth rate (Gil et al., 2006). Undoubtedly, more research is warranted to better understand the potential anabolic effect of A4 on a wider diversity of taxa. However, the available data supports the notion that A4, through its anabolic and proliferative effects, could indeed be implicated in creating a more competitive and resistant phenotype (Cariello et al., 2006; Gil et al., 2007).

Interaction between A4 and leptin: A possible regulation of begging behaviour

Gil et al. (2007) in its comparative study in birds found that larides and rallides have the highest levels of yolk A4. Despite being precocial (feathered at hatching with locomotive activity), these species obtain food from their parents by begging (Eising and Groothuis, 2003). It has been suggested that the high levels of A4 in these nidufuge species, may be related to the need of developing strong begging capacity (Gil et al., 2007). It is possible, at a broader level at least, that A4 could be implicated in the development of begging skills via its interactions with leptin.

Adipose tissue produces a polypeptide hormone named leptin. This polypeptide is the product of the expression of the Ob gene and act as a signal to inform the hypothalamus about the levels of fat reserves stored in the organism. As part of a neuroendocrine adaptation to fasting (Ahima et al., 1996), leptin regulates appetite and energy expenditure (Caro et al., 1996), and modulates the secretion of growth hormone (Carro et al., 1997). High levels of leptin, through its action on receptors located in the hypothalamus, provoke a reduction in food intake (Denbow et al., 2000). Interestingly, in humans, A4 has been identified as a

regulator of leptin secretion. *In vitro* experiments have shown that adipose tissue inhibits its leptin secretion when incubated in the presence of A4 (Piñero et al., 1999). This effect was not mediated by T and was sex specific, with a decrease in leptin production observed in tissue from women but not from men (Piñero et al., 1999). Leptin has also been shown to regulate food intake and growth patterns in poultry (Denbow et al., 2000). Long-term selection for early meat production in domestic hens (*Gallus domesticus*) has resulted in a 2.5-fold increase in age-specific body weight with only a 22.5% increase in digestive efficiency (Denbow, 1999). The observed increase in growth rate seems to reflect a lower sensitivity or lower responsiveness to the feeding inhibiting effects of leptin (Denbow et al., 2000; Cassy et al. 2004). Leptin expression has been detected in liver in chicken embryos as well as in yolk sac (Ashwell et al., 1999). Based on these results, it is reasonable to suggest that A4 may be involved in the control of begging in birds through its positive regulation of body mass, via its actions on leptin secretion (Clark, 2002), and that the effects may differ between sexes (Piñero et al., 1999; Clark, 2002). If A4 decreases the secretion of leptin, which in turn signals the need to increase food intake, it can be hypothesized that high levels of A4 could indirectly lead to an increase in begging behaviour. Additionally, if yolk A4 correlates with chick´s plasma A4 (e.g. *Sula nebouxii*, Dentressangle and Torres, in prep), differential maternal allocation of yolk A4 may influence the begging behaviour of chicks after hatching. This hypothesis has not been tested. Further studies are needed to determine whether begging behaviour is indeed influenced by levels of leptin secretion, and whether the effects of A4 on leptin secretion observed in *in vitro* experiments (Piñero et al., 1999) occurs in animals in the wild.

STIMULATORY EFFECTS ON IMMUNE RESPONSE

Previous studies have shown that T contained in yolk can alter the immune response of chicks. The results however, are inconsistent between studies. Some authors have found a positive relation (Navara et al., 2006), others found a negative one (Muller et al., 2005; Navara et al., 2005), and yet others have found no effect (Andersson et al., 2004). A possible reason for this discrepancy is the different T concentrations used in different studies. As evidenced by the work of Navara et al. (2006), the injection of a small amount of testosterone (0.3 μg) in eggs, enhances the cellular immune response of chicks, whereas higher concentration (3 μg) inhibits it. Another important factor to consider in interpreting these results is the sex of the chick, as androgens can have sex-specific effects on the immune system (Martin, 2000; Muller et al., 2003). A4 enhances the proliferation of various types of cells and has the potential to stimulate the proliferation of thymocytes, unlike testosterone which inhibits it (Yao and Shang, 2005). Functionally mature antigen specific T cells are generated in the thymus, the primary lymphoid organ, and then migrate to peripheral lymphoid tissues where they mediate the protection against invading microbes (Santoni et al., 2000). Two studies have found a positive effect of yolk A4 on the cellular immune response of bird chicks (species?, Gil et al., 2006; *Sula nebouxii;* Dentressangle and Torres, submitted). Similarly, a positive correlation between yolk A4 and IgG concentrations was found in kittiwakes (*Rissa tridactyla)* (Gasparini et al., 2007).

Interestingly, high yolk A4 concentrations are positively related to the relative length of the incubation period and negatively correlated with the relative length of the nesting period (Gil et al., 2007). In general, birds with short incubation periods suffer comparatively higher mortality related to parasitism than nestling with longer incubation periods (Ricklefs, 1992). High yolk A4 levels in species with short incubation times could thus, compromise the development of the immune system so that it is relative immature at hatching (Gil et al., 2007; Ricklefs 1992). Therefore, it is important to consider the effects of A4 on

immunocompetence, as yolk A4 can act as a potential modulator of cellular immunity and thus affect survival.

EFFECTS OF A4 ON DOMINANCE AND AGGRESSIVE BEHAVIOUR

As no information is available for birds, in this section I will principally focused on mammals' studies.

Organizational effects of A4: implications for aggressive behaviour

Studies in various species have shown that A4 is positively correlated with aggressive behaviour in rats (*Rattus norvegicus*) (Christie and Barfield, 1979), spotted hyenas, (*Crocuta crocuta)* (Glickman et al., 1987), lemurs (*Lemur catta)* (Drea et al., 2007), gold fish (*Carassius auratus)* (Poling et al., 2001), and human youngsters (Azurmendi et al., 2006; Ramirez, 2003). In rats, the expression of aggressive behaviour is known to be influenced positively by the vasopressin projections of the bed nucleus of the stria terminalis and the centromedial amygdale (Ferris, 1992), which in turn seem to be activated by A4 (Villalba et al., 1999). Consequently, the administration of A4 reverts the reduction of the vasopressin innervation induced by castration by acting at a neuronal level (Villalba et al., 1999), and therefore has the potential of restoring aggressive behaviour in castrated animals to "normal levels". Contrary to the commonly held view of A4 as a weak androgen, A4 has the capacity to mimic the effects of T on the neuronal web (Villalba et al., 1999; Hagemeyer et al., 2000). For instance, in embryonic hippocampal neurons, both, T and T converted into A4, induce a similar reduction in the expression of β tubulin as part of the maturation of neurons (Hagemeyer et al., 2000). A4 also plays a significant role in the excitatory mechanism of neurons (Schwartz et al., 2002) due to its capacity of modulating Ca^{2+} channels (Machelon et al., 1998). Incidentally, A4 has been shown to enhance memory (Flood et al., 1992), and it has been considered to be useful in the treatment of depression, as it has been shown to

decrease depressive states in rats (Schwartz et al., 2002). This evidence underlines the potential role of androstenedione in modifying neuronal sensitivity and affecting the limbic system. As such, it highlights the relevance of considering the potential impact of A4 on the cognitive and emotional aspects of an organism's life when undertaking behavioural ecology studies.

Behavioural studies

Hyenas have been the subject of many biologists' attention for the extreme masculinisation of the external genitalia shown by females of this species (Glickman et al., 1987). Females are generally larger, more dominant and exhibit more aggressive behaviours than their male counterparts (Glickman et al., 1987; Szykman et al. 2003). In this species, aggression does not seem to be explained by circulating T levels (Goymann et al., 2001; Dloniak et al., 2006). Nevertheless, high ranking females have higher faecal androgen concentrations than low ranking females (Dloniak et al., 2006). Dloniak and collegues found a strong association between maternal faecal androgen concentrations during the second half of gestation and the aggressive behaviour of her cubs, suggesting that a female can pass on her social rank to her cubs by exposing them to corresponding androgen levels during gestation. Unfortunately, the use of cross-reactive antibiodies in this study makes it impossible to safely attribute such effects to one of the two androgens: T or A4. However, the fact that adult female hyenas exhibit higher levels of A4 than T raise the possibility that A4 could be the androgen implicated (Glickman et al., 1987). Additionally, plasma A4 concentrations in female cubs during infancy can be up to four times higher than in male cubs. These sex differences are maintained until the prepubertal stage but disappear during adulthood (Glickman et al., 1987). Thus, it has been hypothesized that in this species, A4 is responsible for both, organizational and activational effects on aggressive behaviour. For example, adult females treated with

antiandrogens while pregnant produced infants with reduced levels of A4 that exhibited less severe sibling aggression during the early postnatal period (Drea unpublished data in Drea, 2007). Similarly, ovariectomised female juveniles exhibit significantly lower aggression toward males as adults (Backer, 1990 in Drea, 2007).

The spotted hyena is not the only mammal to show a sex reversed pattern in terms of dominance. Female dominance over males is frequently observed in strepsirrhine primates from Madagascar (Richard, 1987). Incidentally, females of the ring-tailed lemur, *Lemur catta*, show higher levels of A4 than T throughout the year, with increased differences during the reproductive period in which female-initiated aggression is higher than at any other part of the year (Drea, 2007). The fact that T levels remain at "basal level" and do not show any fluctuation throughout the year, makes it unlikely that T is responsible for changes in female aggressive behaviour. Instead, the androgenic activity observed in this species is most likely due to the direct effects of A4 or, alternatively, to the indirect estrogenic effect of A4 through its conversion into estradiol (Drea, 2007).

A similar role for A4 on social behaviour has been demonstrated in humans, specifically in preschool children (Azurmendi et al., 2006). Since during this prepubertal stage, most androgen production is of adrenal origin, levels of these steroids are low compared to those during puberty and adulthood (Forest, 1989). It has been suggested that during the prepubertal stage androgens are implicated in the initiation and maintenance of aggressive behaviour (Sanchez-Martin et al., 2000). In 5-years-old boys for example, Azurmendi et al. (2006) found a positive relationship between A4 levels and provocative behaviour, and a negative one between A4 levels and victimization behaviour. Boys with high levels of A4 engaged more in aggressive behaviour and were less likely to be the object of peer aggression (Azurmendiet al., 2006). These relationships were not detected in girls: A4 in

girls has instead been positively associated with the expression of anger (Inoff-Germain et al., 1988).

It thus seems possible that in species that exhibit a dominant hierarchy among siblings, A4 could be implicated in its establishment by promoting a dominant role in individuals with high levels of A4 and subordination in those with lower ones. Further research is needed to address this hypothesis.

CONCLUSIONS

Despite the widely accepted notion of A4 as a weak androgen, this steroid has been shown to affect various functions in ways that have important implications for an individual's life. As suggested by Gil and collaborators (2007), A4 has the potential to participate in the production of competitive phenotypes. Through its organizational effects in the brain, A4 may condition the individual, to behave more or less dominant and/or aggressive in a competitive situation. Additionally, through its anabolic effects, A4 can stimulate growth at the muscular and skeleton levels and, in contrast to T, can enhance the immune system. Moreover, the effect of A4 on leptin secretion strongly suggests that A4 can indirectly affect begging behaviour.

Evidence has been presented in favour of a role for A4 in the production of a highly competitive phenotype characterised by a rapid growth rate, a good immune response and high social rank, traits that are expected to contribute to improve survival and ultimately, fitness (Fig 1). Nevertheless, information on A4 and its effects are somehow limited, particularly in birds. Most available data relates to the effects of A4 on aggressive and dominant behaviour in mammals, and thus more information of the effects of this steroid in other taxa is warranted. Furthermore, the results of *in vitro* experiments need to be validated in live animals and in animals in the wild, since some implicated factors are likely to vary.

Further studies on the enzymatic composition of bird eggs would be very useful as they would help in determining whether the observed effects of A4 are indeed due to its direct effects or whether they are related to A4 through its conversion into T or estradiol. The inclusion of A4 in physiological and behavioural models could be a fruitful way to investigate how steroid-driven morphological modifications in the brain can be translated into changes in behaviour, as well as to understand how A4, an androgen with a chemical structure so closely related to that of T can act in different and sometimes, opposite ways.

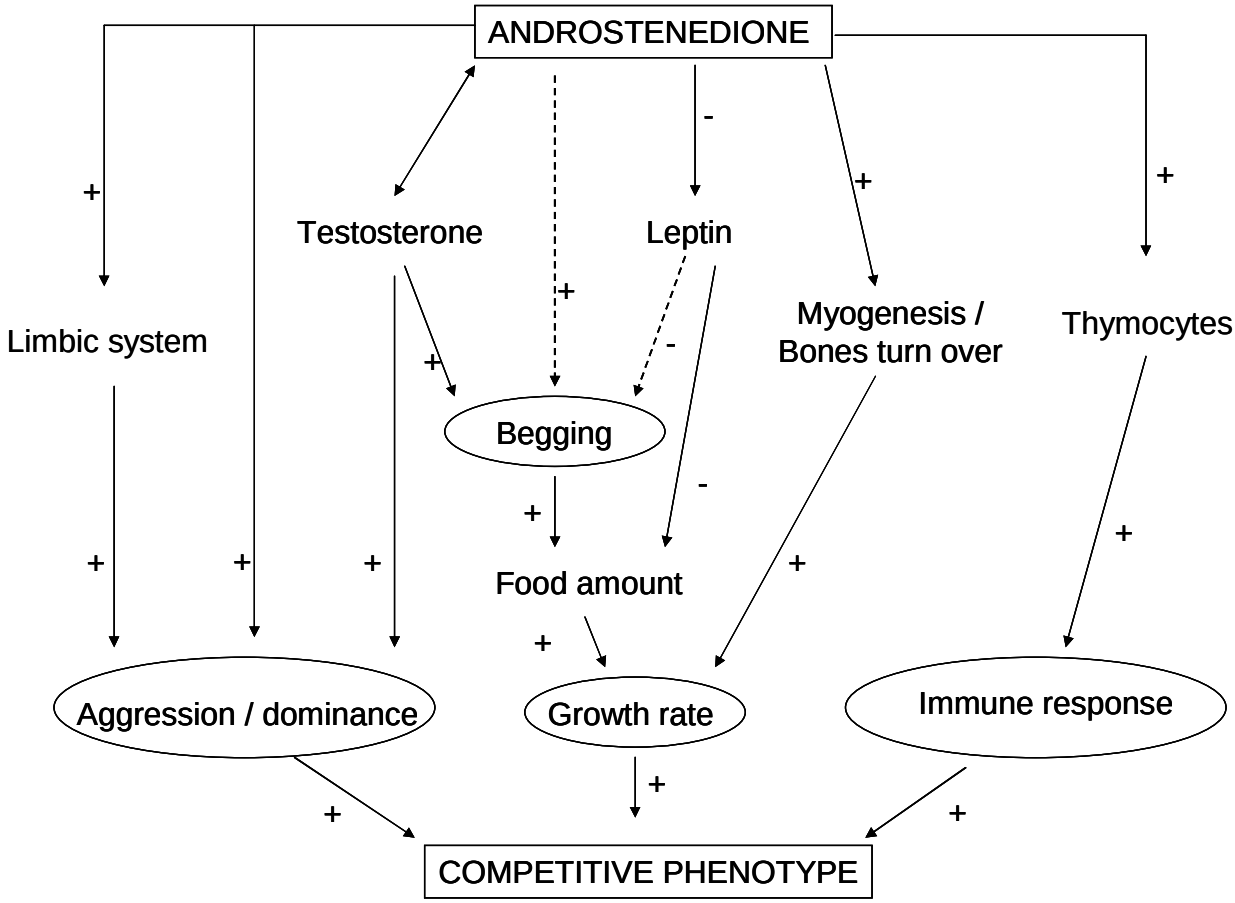

Figure1. Possible pathways for androstenedione (A4) to produce a competitive phenotype. Straight lines represent known effects whereas dash lines represent hypothetical effects. Stimulatory effects are symbolized by (+) and inhibiting effects by (-).

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