UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS
INSTITUTO DE ECOLOGÍA
BIOLOGÍA EVOLUTIVA Y SISTEMÁTICA

SEÑALES DINÁMICAS EN EL BOBO CAFÉ (*Sula leucogaster*): FUNCIÓN, COMPROMISOS FISIOLÓGICOS Y COADAPTACIÓN

TESIS

QUE PARA OPTAR POR EL GRADO DE:
DOCTORA EN CIENCIAS

PRESENTA:

BIBIANA CAROLINA MONTOYA LOAIZA

TUTORA PRINCIPAL DE TESIS: DRA. LAURA ROXANA TORES AVILÉS
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COMITÉ TUTOR: DR. CARLOS RAFAEL CORDERO MACEDO
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DRA. GUILLERMINA ALCARÁZ ZUBLEDÍA
FACULTAD DE CIENCIAS

MÉXICO, D.F. ENERO, 2016

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UNAM POSGRADO
Ciencias Biológicas

COORDINACIÓN

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
Presente

Me permito informar a usted, que el Subcomité de Biología Evolutiva y Sistemática, en su sesión ordinaria del día 21 de septiembre de 2015, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTORA EN CIENCIAS**, del Posgrado en Ciencias Biológicas, de la alumna **MONTOYA LOAIZA BIBIANA CAROLINA** con número de cuenta **511451249** con la tesis titulada **"SEÑALES DINÁMICAS EN EL BOBO CAFÉ (*Sula leucogaster*): FUNCIÓN, COMPROMISOS FISIOLÓGICOS Y COADAPTACIÓN"**, bajo la dirección de la **DRA. LAURA ROXANA TORRES AVILÉS:**

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, D.F. a 15 de enero de 2016

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

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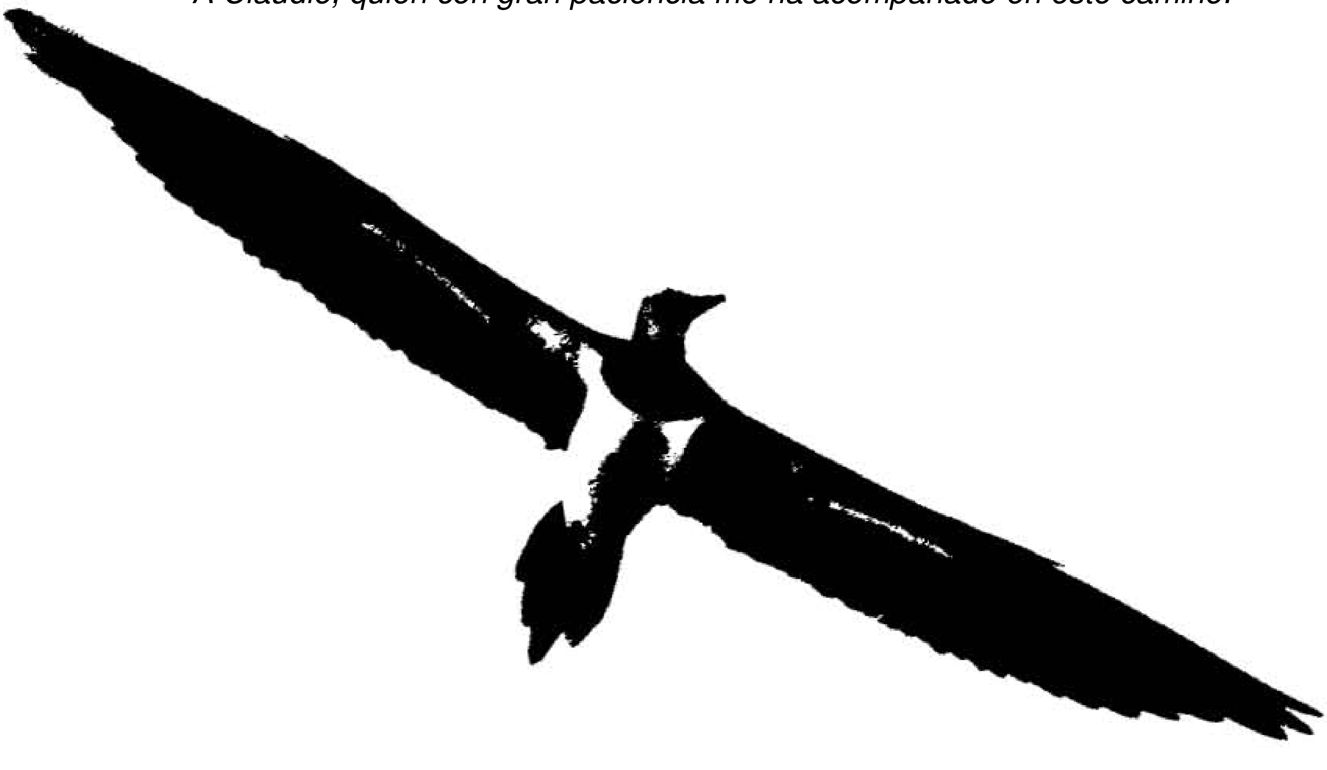

Foto original cortesía de Angélica de la Cruz

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Resumen

Uno de los compromisos evolutivos más estudiados es el que involucra los costos en términos de reproducción futura, que surgen como resultado de la inversión reproductiva actual. Pese a que el concepto de compromisos entre componentes de historia de vida es clave para la teoría, se sabe relativamente poco sobre los mecanismos fisiológicos subyacentes. Los procesos fisiológicos asociados al funcionamiento del organismo durante la reproducción, o los productos resultantes de estos procesos, podrían impactar negativamente el mantenimiento somático. En particular, el aumento en la producción de radicales libres durante la reproducción, podría exponer al individuo a un desbalance oxidante, que al prolongarse, podría dañar biomoléculas como costo próximo de la reproducción. Numerosos estudios han evaluado esta hipótesis, arrojando evidencia mixta en favor y en contra de ella. Debido a los costos asociados a la reproducción, los individuos se ven favorecidos al basar su elección de pareja en la magnitud de los beneficios directos o indirectos que esta puede ofrecer. Sin embargo, esta magnitud no puede ser evaluada de forma directa, lo cual ha promovido la evolución de mecanismos indicadores. Para que las señales de beneficios evolucionen y se mantengan estables, deben imponer costos diferenciales a los portadores dependiendo de su calidad y/o estar asociadas a la condición del portador. El uso de señales dependientes de la condición del emisor no se limita al contexto de la elección de pareja. Durante la interacción padres-cría el nivel óptimo de inversión en cuidados parentales es diferente para las dos partes, por lo cual se ha propuesto que, señales emitidas por la cría podrían modular la inversión de los padres. Debido a que la tasa de entrega de alimento por parte de los padres y la tasa de emisión de señales de solicitud por parte de las crías tienen efectos epistáticos sobre la adecuación de la cría, la ocurrencia de un proceso de coadaptación entre estas dos características ha sido sugerida. Usando como modelo de estudio al bobo café (*Sula leucogaster*), una especie de larga vida con cuidado bi-parental obligado y prolongado, en el capítulo II de esta tesis se evaluó si los niveles de estrés oxidante (1) se relacionan con la inversión en ornamentos coloridos, (2) limitan el esfuerzo en la reproducción, y (3) son un costo próximo de la reproducción. En el capítulo III se evaluó si características coloridas durante el cortejo, informan a la pareja reproductiva sobre beneficios directos o indirectos. En el capítulo IV se evaluó si el color del gular en machos y hembras es una señal dinámica. Finalmente, en el capítulo V se evaluó la posible existencia de co-adaptación entre la tasa de alimentación de los padres y la tasa de solicitud de la cría.

En el capítulo II se encontró que machos y hembras con gulares más coloridos presentan mayor daño oxidante en lípidos y mayores niveles de radicales libres al acercase la puesta. Las hembras con mayores niveles de radicales libres en cortejo, pusieron primeros huevos más pequeños y criaron pollos que ganaron menos peso, y las hembras con mayor daño en lipidos en cortejo cuidaron menos tiempo de las crías. Contrario a lo esperado, los niveles de daño oxidante en lípidos no incrementaron con el esfuerzo reproductivo, mientras que, la produción de radicales libres fue mayor en cortejo que en crianza. Los resultados sugieren que en el cortejo un incremento en el daño oxidante y en la producción de radicales libres podría ser consecuencia de la inversión en ornamentos coloridos y en la puesta; mientras que

altos niveles de daño oxidante o radicales libres podrían limitar la inversión reproductiva de las hembras.

En el capítulo III, usando un diseño experimental de intercambio de puestas para separar efectos genéticos de efectos ambientales, se encontró que machos con gulares más coloridos tienen crías con mayor tasa de crecimiento estructural, atienden más tiempo a sus crías y las alimentan más, favoreciendo una mayor ganancia de peso. No se encontró evidencia de que las hembras apareadas con machos más coloridos aumenten su inversión en la puesta o en la crianza. Los resultados indican que el color del gular de los machos es una señal de beneficios directos e indirectos para las hembras.

En el capítulo IV se encontró que, aunque machos y hembras difieren en el color que exhiben en la piel del gular, esta coloración varía de forma similar en los dos sexos a lo largo de la temporada reproductiva. Cuando se consideró la capacidad para percibir los cambios en color, solo los cambios intraindviduales en la coloración de los machos a lo largo del evento reproductivo mostraron alguna probabilidad de ser percibidos por otros miembros de la especie; mientras que, durante el cortejo las diferencias de color entre individuos tanto en machos como en hembras podrían ser percibidas por co-específicos.

Finalmente, en el capítulo V además de encontrar una correlación positiva entre las alimentaciones de los padres y las solicitudes de las crías, las madres de bobo café, presentaron una correlación negativa entre su tendencia a alimentar y la tendencia a solicitar de sus crías genéticas. La ocurrencia de esta correlación negativa sugiere que combinaciones de madres que alimentan a tasas altas y crías que solicitan poco, así como madres que alimentan a tasas bajas y crías muy solicitadoras han resultado favorecidas por las presiones de selección. La ruptura de esta co-variación afectó la capacidad inmune de los machos y probablemente de las hembras. Los resultados sugieren la ocurrencia de un proceso de coadaptación madre-cría.

En conclusión, los resultados de esta tesis sugieren la existencia de un compromiso entre la inversión reproductiva en cortejo y en crianza mediado por el estrés oxidante. Por otra parte, los resultados señalan que la coloración de la piel del gular en el bobo café es una señal sexual dinámica, dependiente de carotenoides, que impone costos oxidantes, y que en los machos señala beneficios directos e indirectos para las hembras, por consiguiente podría ser una señal honesta usada durante la elección femenina y probablemente durante la elección mutua. Finalmente, los resultados sugieren una interdependencia entre la provisión materna y la solicitud de las crías que posiblemente ha favorecido la selección de combinaciones específicas de estas dos características.

Dynamic signals in the brown booby (*Sula leucogaster*): Function, physiological trade-offs and co-adaptation

Abstract

One of the most studied evolutionary trade-off is the future reproduction cost entailed by current reproductive investment. Despite the theoretical importance of the trade-off concept in life-history theory, physiological mechanisms underlying such trade-offs are still largely unknown. Physiological processes linked to organism functioning during reproduction, or its byproducts, might exert a negative impact on the somatic maintenance processes. Particularly, an increase in the free radical production may expose individuals to an oxidative unbalance that extended may threaten bio-molecules.

Several studies have evaluated such hypothesis obtaining both, evidence for and against. Considering the costs reproduction entails, individuals result favored by evaluating the magnitude of direct or indirect benefits a potential mate can offer. However, the magnitude of the benefits cannot be directly evaluated during mate choice promoting the evolution of signaling mechanisms. In order to evolve and stay stable signals should impose differential cost to bearers depending on their condition, or be associated with such condition. Condition-dependent signals are not used exclusively in the context of mate choice. During parents-offspring interactions the optimal level of investment differs between the parents and the offspring, thus offspring signals of condition have been proposed to modulate parental investment. Parental provisioning and offspring begging have epistatic effects on offspring fitness, thereby the occurrence of a co-adaptation process between these two traits has been proposed. Using as a model of study the brown booby (*Sula leucogaster*), a long-lived seabird with prolonged and obligated bi-parental care, in chapter II of this thesis we evaluated whether oxidative stress (1) is related to investment in colorful ornaments, (2) constrains the reproductive effort, and (3) is a proximate cost of reproduction. In chapter III, we evaluated if colorful traits during courtship provide information about the benefits a reproductive mate can offer. In chapter IV, we evaluated the occurrence of variation throughout the reproductive season in male and female gular color. Finally, in chapter IV the existence of a co-adaptation between parents provisioning and offspring begging was evaluated.

In chapter II, we found that males and females with more colorful gular had more oxidative damage in lipids, and higher levels of free radicals as laying date approached. Females with higher level of free radicals at courtship laid smaller first eggs and reared offspring with lower body mass gain, and females with more oxidative damage in lipids at courtship attended their offspring during less time. Contrary to expected, levels of oxidative damage in lipids did not increase with the reproductive effort, whereas free radical production was higher during courtship than parental care. These results suggest that during courtship increased oxidative damage and free radical production might be a cost of the investment in colorful ornaments and clutch establishment, while higher levels of oxidative damage or free radicals may constrain female reproductive investment.

In chapter III, using a cross-fostering experiment to separate genetic from environmental effects, we found that males with more colorful gular had offspring with higher structural growth, attended their offspring longer, and fed them more promoting higher body mass gain. Females paired with more colorful males did not

increase their clutch or parental investment. These results suggest that male gular color is a signal of direct and indirect benefits for females.

In chapter IV, despite the difference in gular color by sex, we found this coloration to change throughout the reproductive season in a similar way in both, males and females. When visual system was considered, only intra-individual changes in male gular color through the reproductive event showed some probability of being perceived by other members of the species. Between-individuals variation in gular color during courtship in both, males and females may be perceived by conspecifics.

Finally, in chapter V, besides a positive correlation between parental feeding and offspring begging, we found mother provisioning to be negatively correlated with genetic offspring begging. This negative genetic correlation suggests that combinations of mothers with high provisioning rate and offspring with low begging rate, as well as, mothers with low provisioning rate and offspring with high begging rate have been favored by selective pressures. Disrupting this co-variation affected immune response of fathers and probably mothers. These results suggest the occurrence of a mother offspring co-adaptation.

In conclusion, our results suggest the occurrence of a trade-off between reproductive investment during courtship and parental care, mediated by oxidative stress. Moreover, results suggest that gular skin color in the brown booby is a dynamic sexual trait, carotenoid dependent, which exerts oxidative costs and in males signals direct and indirect benefits for females. Therefore, this coloration may be an honest signal used during female, and probably mutual, mate choice. Additionally, male gular color signals direct and indirect benefits for females. Finally, results also suggest interdependency between maternal provisioning and offspring demand, which has possibly favored selection of specific combinations of these two traits.

CAPÍTULO I

Introducción General

Foto original cortesía de Natalia Lfshitz

INTRODUCCIÓN GENERAL

Compromisos entre historias de vida

El principio fundamental de la *teoría de historias de vida*, se basa en la existencia de un compromiso entre distintas funciones asociadas a la adecuación, que ocurre cuando un cambio benéfico en una función vital está vinculado a un cambio perjudicial en otra (Stearns 1989, 1992). Probablemente, el compromiso más estudiado es el costo en términos de supervivencia y reproducción futura, que resulta de la inversión reproductiva actual (Stearns 1989). Este compromiso puede ocurrir a nivel genotípico, fenotípico o fisiológico, ya que esto conecta el genotipo con el fenotipo (Stearns 1989). Tradicionalmente, se ha propuesto que la asignación de recursos a los sistemas vitales, es optimizada con respecto a las demandas y la etapa específica del ciclo de vida, pero debido a que la cantidad de recursos para invertir es limitada, un aumento en la inversión en una función, disminuye los recursos disponibles para otras funciones (Stearns 1992; Reznick *et al.* 2000; Ricklefs and Wikelski 2002). Por una parte, la experiencia reproductiva previa y la condición fisiológica actual del individuo podrían limitar la asignación de recursos a la reproducción (Curio 1983); mientras que por otra parte, la esperanza de vida reproductiva residual podría restringirla (Williams 1966). Recientemente, la comprensión de los compromisos entre historias de vida ha pasado de la búsqueda de correlaciones negativas entre componentes, a la búsqueda de los mecanismos próximos que subyacen a las relaciones funcionales negativas entre ellos (Zera y Harshman 2001; Flatt y Heyland, 2011). En esta búsqueda, se ha considerado la posibilidad de que los procesos fisiológicos asociados al funcionamiento del organismo durante la reproducción, puedan infligir directamente daño somático y producir un impacto negativo sobre otras funciones vitales, incluso en ausencia del compromiso de recursos inicialmente propuesto como base del dilema (Harshman y Zera 2007; Dowling y Simmons 2009; Monaghan *et al.* 2009).

Estrés oxidante como costo de la reproducción

El estrés oxidante ha sido propuesto como mecanismo subyacente a los costos fisiológicos asociados a la inversión reproductiva, independiente a la ocurrencia del compromiso de recursos tradicionalmente considerado como costo de la reproducción (Alonso-Alvarez *et al.* 2004). Esta hipótesis se deriva de la *teoría del envejecimiento por radicales libres*, de acuerdo con la cual, los radicales libres no neutralizados generan reacciones que podrían dañar las moleculas del organismo conduciendo paulatinamente a envejecimiento somático (Harman 1972). Los radicales libres, son átomos o compuestos que presentan uno o más electrones desapareados en su capa externa, lo que los hace altamente reactivos por su tendencia a captar electrones de otros átomos, generando una reacción en cadena (Halliwell y Gutteridge 2007). El proceso de respiración celular es considerado como la fuente principal de producción de radicales libres (Harman 1972). La teoría del envejecimiento por radicales libres propone un vínculo entre tasa de envejecimiento y tasa metabólica, donde un incremento en la tasa metabólica asociado por ejemplo a la inversión en la reproducción, generaría una sobreproducción de radicales libres que al permanecer no neutralizados, podría dañar biomoléculas y promover el envejecimiento (Beckman y Ames 1998; Dowling y Simmons 2009).

Como sub-producto de las reacciones oxidación-reducción (redox) implicadas en la producción de adenosín trifosfato (ATP), la respuesta inmune y la respuesta antioxidante, se liberan agentes oxidantes, llamados de manera genérica radicales libres (Halliwell y Gutteridge 2007; Dowling y Simmons 2009). Los radicales libres cumplen un papel importante en la señalización celular, p. ej., regulan el tono vascular, la adhesión de plaquetas, la división celular y la muerte celular programada (Halliwell y Gutteridge 2007). Cuando ocurre una sobreproducción de radicales libres, el organismo tiene la capacidad de neutralizar su acción haciendo uso de antioxidantes de origen enzimático endógeno como, la superóxido dismutasa, la catalasa y la glutatión peroxidasa; y exógeno como, el glutatión reducido, las vitaminas C y E y algunos pigmentos como los carotenoides. Estos antioxidantes pueden actuar de manera específica, complementaria o sinérgica (Halliwell y Gutteridge 2007; Finkel y Holbrook 2000). Cuando la sobreproducción de radicales libres supera la capacidad de defensas antioxidantes para neutralizarlos, puede generarse daño en substratos biológicos importantes como lípidos, carbohidratos, proteínas y ADN, comprometiendo el correcto funcionamiento fisiológico (Halliwell y Gutteridge 2007). Sin embargo, incluso si los agentes oxidantes alcanzan a infligir algún daño, este puede ser reparado por el sistema antioxidante (Halliwell y Gutteridge 2007). El deterioro en biomoléculas resultante de esta acción de los radicales libres no neutralizados y de la falla en los procesos de reparación, ha sido propuesto como mecanismo próximo que modula los compromisos entre características de historias de vida (Monaghan *et al.* 2009, Metcalfe y Alonso-Álvarez 2010).

El aumento en el consumo de oxígeno y por ende, la producción de radicales libres se encuentran asociados a una alta tasa metabólica o a un alto gasto energético diario, y el incremento en estos dos parámetros durante el esfuerzo reproductivo (p. ej. Deerenberg *et al.* 1995; Nilsson 2002), podría favorecer la producción de radicales libres, exponiendo al individuo al riesgo de sufrir un desbalance oxidante y subsecuente daño como costo próximo de la reproducción (Alonso-Alvarez *et al.* 2004, Monaghan *et al.* 2009; Metcalfe y Alonso-Álvarez 2010). Sin embargo, debido a la flexibilidad de la mitocondria para funcionar de manera desacoplada, la relación incremento en la tasa metabólica – producción de radicales libres podría no ser tan directa como se había pensado (p. ej. Salin *et al.* 2015). Durante la fosforilación oxidante, las proteinas que transportan electrones, vía reacciones redox, pueden desacoplarse permitendo a los protones pasar la membrana de la mitocondria generando calor sin producir ATP, reduciendo de esta forma la producción de radicales libres a expensas de la producción de ATP (Criscuolo *et al.* 2005; Speakman *et al.* 2004).

Al margen de la existencia de una relación directa entre incremento en la tasa metabólica y aumento en la producción de radicales libres, se ha propuesto otra vía por medio de la cual el organismo podría quedar expuesto a la acción de agentes oxidantes como consecuencia de la inversión reproductiva. La activación del sistema inmune observada durante el esfuerzo reproductivo (p. ej. Fedorka *et al.* 2004; Hanssen *et al.* 2005), también podría generar un desbalance oxidante en el organismo, con los riesgos de daño asociados (Dowling y Simmons 2009). El sistema inmune puede utilizar, entre otros mecanismos, reacciones redox durante la defensa contra el ataque de patógenos, p. ej. durante la fagocitosis, los fagocitos producen especies de oxígeno reactivo (ROS por su sigla en inglés) vía explosiones oxidativas rápidas para atacar a los invasores (Bender *et al.* 2005). Sin embargo, debido a que los radicales libres no ejercen una acción específica sobre agentes patógenos, sino general sobre biomoléculas, esta reacción oxidante desencadenada para defender al organismo podría también dañarle (von Schantz *et al.* 1999; Dowling y Simmons 2009). Por medio de cualquiera de estas dos vias, un incremento en la tasa metabólica o una activación de la respuesta inmune, la variación individual en la habilidad para evitar o manejar un exceso de radicales libres no neutralizados durante el esfuerzo reproductivo podría modular el costo de la reproducción, y a nivel fisiológico mediar compromisos entre diferentes componentes de historia de vida (e.g. la inversión en ornamentos sexuales versus inversión en cuidado parental).

La evidencia acerca de la existencia de un compromiso entre inversión reproductiva y daño oxidante es controversial. Los estudios iniciales que abordaron esta idea, principalmente midieron el componente antioxidante del balance, encontrando que el incremento en el esfuerzo reproductivo reducía la capacidad antioxidante de los individuos (Alonso-Alvarez *et al.* 2004, Wiersma *et al.* 2004). Sin embargo, una reducción en la capacidad antioxidante no necesariamente indica un incremento en el daño oxidante. La capacidad antioxidante también podría disminuir después de haber neutralizado efectivamente la sobreproducción de radicales libres, o en respuesta a una reducción en la producción de radicales libres como resultado de la regulación homeostática (Monaghan *et al.* 2009). Estudios posteriores que se han enfocado en evaluar la ocurrencia de daño oxidante en biomoléculas, han aportado resultados en favor y en contra de las predicciones originales, dependiendo del substrato biológico evaluado (plasma, células hepáticas, renales, cardiacas), el sexo del individuo y el diseño del estudio (comparación de individuos reproductivos contra no reproductivos, o manipulación del nivel del esfuerzo reproductivo). En términos generales, la comparación de individuos reproductivos contra no reproductivos, ha sugerido que los individuos reproductivos presentan menor nivel de daño oxidante en comparación con sus contrapartes no reproductivas (p. ej. Garrat et al. 2013, Aloise-King *et al.* 2013, Costantini *et al.* 2014, Wegmann *et al.*

2015); mientras que, la evaluación de la relación entre la magnitud del esfuerzo reproductivo, considerando solamente individuos reproductivos, y el daño oxidante, ha sugerido una relación positiva entre estas dos variables, a mayor esfuerzo reproductivo mayor daño oxidante (p. ej. Bergeron *et al.* 2011, Wilson *et al.* 2012, Xu *et al.* 2013). Un meta-análisis realizado sobre los resultados de los estudios conducidos hasta 2014 sobre el costo oxidante de la reproducción, motivó la sugerencia de la existencia de un mecanismo protector, hipótesis del *escudo oxidante*, contra el daño oxidante durante la reproducción (Blount *et al.* 2015). De acuerdo con la hipótesis del *escudo oxidante*, debido a que el incremento en la inversión reproductiva se asocia a un aumento en el daño oxidante, las hembras en particular, activan una respuesta fisiológica que favorece la disminución en los niveles de daño (Blount *et al.* 2015). Así, al pasar de un estado no reproductivo a un estado reproductivo, esta respuesta protectora disminuye la exposición a los potenciales efectos adversos de un desbalance oxidante, en las madres y en las crías vía efectos maternos (Blount *et al.* 2015). Aunque la hipótesis fué originalmente propuesta para el caso de las hembras en particular, podría esperarse que en especies con cuidado bi-parental obligado y prolongado, el mecanismo del escudo oxidante funcionara de manera similar para ambos sexos. Aun más, en especies con cuidado bi-parental costoso, se podría predecir una preferencia durante el cortejo por individuos más capaces de protegerse del daño oxidante asociado al esfuerzo reproductivo.

Elección femenina

El valor del evento reproductivo actual para machos y hembras, y en consecuencia, el valor marginal de futuros eventos reproductivos puede ser significativamente diferente. Mientras que, los machos no requieren enfrentar la limitante asociada a la cantidad, temporalidad y movilidad de sus gametos sexuales, las hembras se enfrentan a esta restricción; como resultado, los valores óptimos de la inversión reproductiva son diferentes para los dos sexos (Bateman 1948). De forma tradicional se ha propuesto que, las presiones impuestas por estas diferencias han favorecido la selectividad de las hembras al momento de elegir una pareja reproductiva y el uso de ornamentos y/o armamentos en los machos para ganar oportunidades reproductivas, competir contra otros machos por acceso a éstas, o incluso forzar a las hembras a invertir en un evento reproductivo (Darwin 1871, Andersson 1994, Holland y Rice 1998). Es importante señalar que, aunque esta es la aproximación tradicional, en diferentes especies se ha encontrado que un proceso de elección mutua podría haber favorecido la evolución de ornamentos sexuales en las hembras y selectividad en los machos durante la elección de pareja (Massaro *et al.* 2003; Griggio *et al.* 2009; Nolan *et al.* 2010; Edward y Chapman 2011).

La *elección femenina* es reconocida como una de las principales fuerzas que ha promovido la evolución de ornamentos extravagantes en machos (Darwin 1871, Andersson 1994). Debido a que, durante el cortejo, la condición de un inividuo y su capacidad para invertir en un evento reproductivo no pueden ser evaluadas directamente, las hembras usan características sexuales como indicadores de condición (Kodric-Brown y Brown 1984). La evolución de ornamentos vistosos ha sido explicada por la explotación de sesgos sensoriales pre-existentes que evolucionaron en contextos diferentes a la elección de pareja, como p. ej. forrajeo (Ryan y Rand 1993). Por otra parte, el mantenimiento de estas características se ha explicado por la obtención, de beneficios directos (para el receptor de la señal) o indirectos (a través de su descendecia), tras aparearse con individuos más ornamentados. Los beneficios directos obtenidos podrían ser mejores regalos nupciales, mejores territorios, menor probabilidad de contagio de enfermedades o, de acuerdo con la *hipótesis del buen padre* mejores cuidados parentales (Hoelzer 1989; Wolf *et al*. 1997). Mientras que, los beneficios indirectos podrían ser: (i) *Hijos atractivos*, en caso de que la variación en el atractivo de ornamentos tenga un componente genético y a su vez, se correlacione genéticamente con la preferencia del sexo opuesto por la característica, por tanto, los hijos de individuos más ornamentados, serán más atractivos y las hijas preferirían machos con ornamentos más atractivos, generando un proceso de *selección desbocada* (Fisher 1930). (ii) *Buenos genes*, en caso de que los ornamentos reflejen la calidad genética y viabilidad del portador, y los hijos de individuos más ornamentados hereden la viabilidad y calidad genética del padre (Zahavi 1975). El tipo de beneficios que mantiene la expresión de ornamentos en una especie determinada, depende de las caraterísticas del sistema de apareamiento y de la historia de vida de la especie en cuestión (Kokko y Monaghan 2001; Kokko y Johnstone 2002; Kraaijeveld *et al.* 2007).

En especies en las que las oportunidades de apareamiento extra-pareja son escasas, el cuidado parental es prolongado y la contribución de los dos padres es indispensable para conseguir que la cría sobreviva, los modelos teóricos predicen que las hembras tenderán a preferir aparearse con machos que provean beneficios directos (Hoelzer 1989, Kokko 1998; García-Navas *et al.* 2012). Mientras que, cuando existe una alta oportunidad de cópulas extra-pareja, el esfuerzo parental del macho no es necesario durante la crianza y las hembras no se ven beneficiadas al acceder a territorios de los machos, se espería en teoría una preferencia de las hembras por beneficios indirectos, siempre y cuando la señal que indique estos beneficios sea heredable y se correlacione con la adecuación de las crías (Zahavi 1975, Kokko *et al.* 2002; Karino *et al.* 2005).

Debido al potencial de los ornamentos sexuales para indicar de manera confiable beneficios directos o indirectos, la inversión reproductiva de las hembras podría ser sensible a ellos de una manera adaptativa (Mousseau & Fox 1998). La *hipótesis de la asignación diferencial* plantea que la estimulación ofrecida por la intensidad de los ornamentos sexuales masculinos, provoca en las hembras una respuesta de incremento en la inversión reproductiva, que podría tener consecuencias sobre el fenotipo de las crías (Burley 1986, 1988; Michl *et al.* 2005, Rutstein, Gilbert & Tomkins 2005). Esta influencia que el fenotipo o el ambiente de la madre puede ejercer sobre el fenotipo de la cría es conocida como *efecto materno* (Mousseau & Fox 1998; Noguera *et al.* 2013). El escenario particular propuesto por la hipótesis de la asignación diferencial es un caso de efecto materno, en el cual el atractivo del macho hace parte del ambiente experimentado por la madre, y tiene un efecto potencial sobre el fenotipo de la cría, por vía de la inversión reproductiva de la madre (Gilbert *et al.* 2012). En aves, los ornamentos sexuales de los machos han mostrado estimular la inversión de las hembras en el huevo (Dentressangle *et al.* 2008; Groothuis y Schwabl 2008) y los polluelos (Limbourg *et al.* 2004; Johnsen *et al.* 2005). La asignación diferencial de recursos por parte de las hembras, durante la inversión reproductiva, en respuesta a los ornamentos masculinos, ha mostrado incluso tener efectos en la expresión de ornamentos sexuales de las crías dependientes de la condición durante la adultez (Gustafsson *et al.* 1995; Wolf *et al.* 1997; Strasser y Schwabl 2004).

Ornamentos dependientes de la condición

La condición de un individuo, es decir, su capacidad para mantener el funcionamiento óptimo de los procesos vitales (Hill 2011), determina la inversión que éste puede hacer en un evento reproductivo, y por consiguiente, los beneficios que puede ofrecer a una pareja reproductiva (Andersson 1994). De acuerdo con esta idea, la evidencia experimental sugiere que los ornamentos son más sensibles que otras características fenotípicas al impacto de enfermedades (Zuk *et al.* 1990). Los ornamentos sexuales son parte de la inversión reproductiva de un individuo, y de acuerdo con la *hipótesis del handicap*, para evolucionar en señales indicadoras de beneficios, requieren reflejar la condición actual del inidividuo o generar costos diferenciales en función de ésta (más altos en cuanto más pobre sea la condición del individuo; Zahavi 1975, Grafen 1990, Iwasa *et al.* 1991). Como resultado de este proceso, la capacidad de un individuo para enfrentar los costos asociados al mantenimiento de un ornamento elaborado determinaría la inversión óptima en la característica, asegurando su valor como señal honesta de calidad genética y/o adaptación local (Zahavi 1975, Grafen 1990, Iwasa *et al.* 1991). La idea de que las señales dependientes de la condición sirven como indicadores honestos de beneficios ha mostrado consistencia lógica (Grafen 1990; Iwasa *et al.* 1991) y ha recibido extenso apoyo empírico (revisado en Johnstone 1995).

Los ornamentos sexuales coloridos han sido propuestos como señales honestas de la condición del individuo, debido al costo fisiológico asociado a la producción y mantenimiento de algunos colores (von Schantz *et al.* 1999). En particular, se ha sugerido que el mecanismo de producción de coloraciones dependientes de carotenoides podría favorecer su honestidad. Los carotenoides son compuestos orgánicos terpenoides, que se dividen entre aquellos que son hidrocarbonos puros (carotenos) y aquellos que contienen oxígeno (xantofilas), su estructura química hace que absorban la luz en longitudes de onda corta como azul y violeta, y la reflejen en longitudes complementarias a las absorbidas, dependiendo del número de dobles enlaces en cadena de la molécula la reflectancia podría ser en rojo, anaranjado o amarillo (Fox 1979). El hecho de que los carotenoides no puedan ser sintetizados *de novo* por los vertebrados, los convierte en un recurso limitado (Olson y Owens 1998). Considerando que la dieta es la unica vía para obtenerlos, los buenos forrajeadores y los individuos que logran extraerlos más eficientemente durante la digestion tendrían acceso privilegiado a estos pigmentos (Senar y Escobar 2002; Karino *et al.* 2005; Casagrande *et al.* 2006). Por otra parte, estas moléculas parecen tener un papel importante en la respuesta inmune, actuando como inmuno-estimulantes (McGraw *et al.* 2003), y en la respuesta antioxidante, actuando como antioxidantes directos, aunque la evidencia al respecto es debatible, o más probablemente como protectores de otros antioxidantes sin funciones pigmentarias (Costantini 2008; Pérez-Rodríguez 2009). Las funciones de los carotenoides en las respuestas inmune y antioxidante, podrían se inter-dependientes, teniendo en cuenta que, la activación de la respuesta inmune incrementa el estrés oxidante (Costantini y Moller 2009). De acuerdo con lo anterior, se ha encontrado que la activación del sistema inmune induce daño oxidante y al mismo tiempo reduce la expresión de ornamentos dependientes de carotenoides (p. ej., Torres y Velando 2007). Recientemente, se ha sugerido un mecanismo adicional subyacente a la honestidad de las señales dependientes de carotenoides, la participación directa o indirecta de estas moléculas en la producción de vitamina A (Hill y Johnson 2012). Sin embargo, la retroalimentación negativa para el mantenimiento de altos niveles de carotenides, cuando los niveles de vitamina A son altos también, parece no ser suficientemente fuerte como para promover la honestidad de la señal (Navarro *et al.* 2010).

Los ornamentos dependientes de carotenoides podrían ser indicadores honestos de la condición del individuo, incluso si los carotenoides no cumplen directamente una función antioxidate o inmuno-estimulante (von Schantz *et al.* 1999). Debido a su estructura química estos compuestos son fácilmente oxidables y podrían perder capacidad pigmentaria como resultado de su oxidación. Adicionalmente, al oxidarse los carotenoides pueden generar productos tóxicos, e inluso, en contexto benignos, la suplementación con carotenoides ha mostrado tener efectos perjudiciales sobre la reprodución futura (Vinkler y Albrecht 2010; Simons *et al.* 2014). Exhibir ornamentos dependientes de carotenoides podría reflejar la condición inmune o antioxidante del individuo, así como su capacidad para lidiar con la potencial toxicidad de estas moléculas.

Coadaptación madre-cría

La inversión reproductiva puede generar costos *intra-individuales*, debidos a una reducción en la probabilidad de sobrevivir para un evento reproductivo futuro y también, *inter-generacionales*, por una dismunución en la probabildad de que las crías sobrevivan para reproducirse (Stearns 1989). Los costos intergeneracionales derivados de la reproducción, hacen que la inversión óptima en un evento reproductivo sea diferente para padres y crías, por lo cual tradicionalmente se ha propuesto que existe un *conflicto entre padres e hijos* sobre el nivel óptimo de inversión en cuidados parentales (Trivers 1974). Mientras que, los padres deben limitar la inversión para no impactar su automantenimiento y por consiguiente, futuras oportunidades reproductivas, las crías por su parte, buscan obtener la mayor cantidad posible de inversión parental para incrementar su probabilidad de sobrevivir y reproducirse. Dado que los padres obtienen beneficios en términos de adecuación por criar descendecia que sobreviva y se reproduzca, se ha propuesto que la interacción padres-cría, está sujeta a una doble presión de selección. Por una parte, se ha sugerido la existencia de una presión de selección sobre la tasa de entrega de cuidados parentales y, por otra, sobre la tasa de solicitud de estos cuidados, esta doble presión, favorece combinaciones cuidados-solicitudes con balances costos-beneficios óptimos para las dos partes (Wolf y Brodie 1998). Esta hipótesis supone la existencia de un componente genético en la variación asociada a la entrega de alimento y a la solicitud de éste, y propone ir más allá de la aproximación del *gambito fenotípico* (Grafen 1991), de acuerdo con la cual, la interacción padres-cría puede ser entendida desde una perspectiva puramente fenotípica (Smiseth *et al.* 2008; Kölliker *et al.* 2000).

De acuerdo con la hipótesis de la *coadaptación madre-cría*, el doble proceso de selección daría lugar a una coadaptación entre la disponibilidad para proveer cuidados por parte de la madre (e.g. alimentaciones) y la tendencia a solicitar por parte de la cría, y debido a que estas dos características tendrían efectos epistáticos sobre la adecuación de la cría, se esperaría que ocurriera una selección correlacional sobre ellas (Agrawal *et al.* 2001; Wolf y Brodie 1998). En consecuencia, además de la correlación fenotípica entre la tasa de solicitudes y la tasa de alimentaciones, se predice también la existencia de una correlación genética entre estas dos características (Wolf y Brodie 1998), y más interesante aún, se propone que el signo de la correlación podría ser diferente a nivel fenotípico y genotípico en función de si las presiones de selección son más intensas en los padres o en las crías (Kölliker *et al.* 2005; Fig. 1). Si la presión de selección recae principalmente sobre la tasa de solicitud de alimentaciones, la correlación genética reflejará la adaptación de las crías al nivel de alimentaciones que proveen los padres, dado que solo las crías de padres que proveen a altas tasas podrían permitirse solicitar a altas tasas, resultando en una correlación positiva a nivel genotípico (Kölliker *et al.* 2005; Fig. 1). Sin embargo, si la presión de selección actúa principalmente sobre los cuidados parentales, la correlación genética reflejará la adaptación de los padres al nivel de demanda de las crías. En consecuencia, las combinaciones favorecidas serán por una parte, padres que alimentan a tasas altas con crías poco demandantes, porque evitará inversión parental excesiva; y por otro lado, crías solicitadoras con padres poco proveedores, porque asegurará un nivel adecuado de provisión, conduciendo a una correlación genética negativa entre las dos características (Kölliker *et al.* 2005; Wolf y Brodie 1998; Fig. 1).

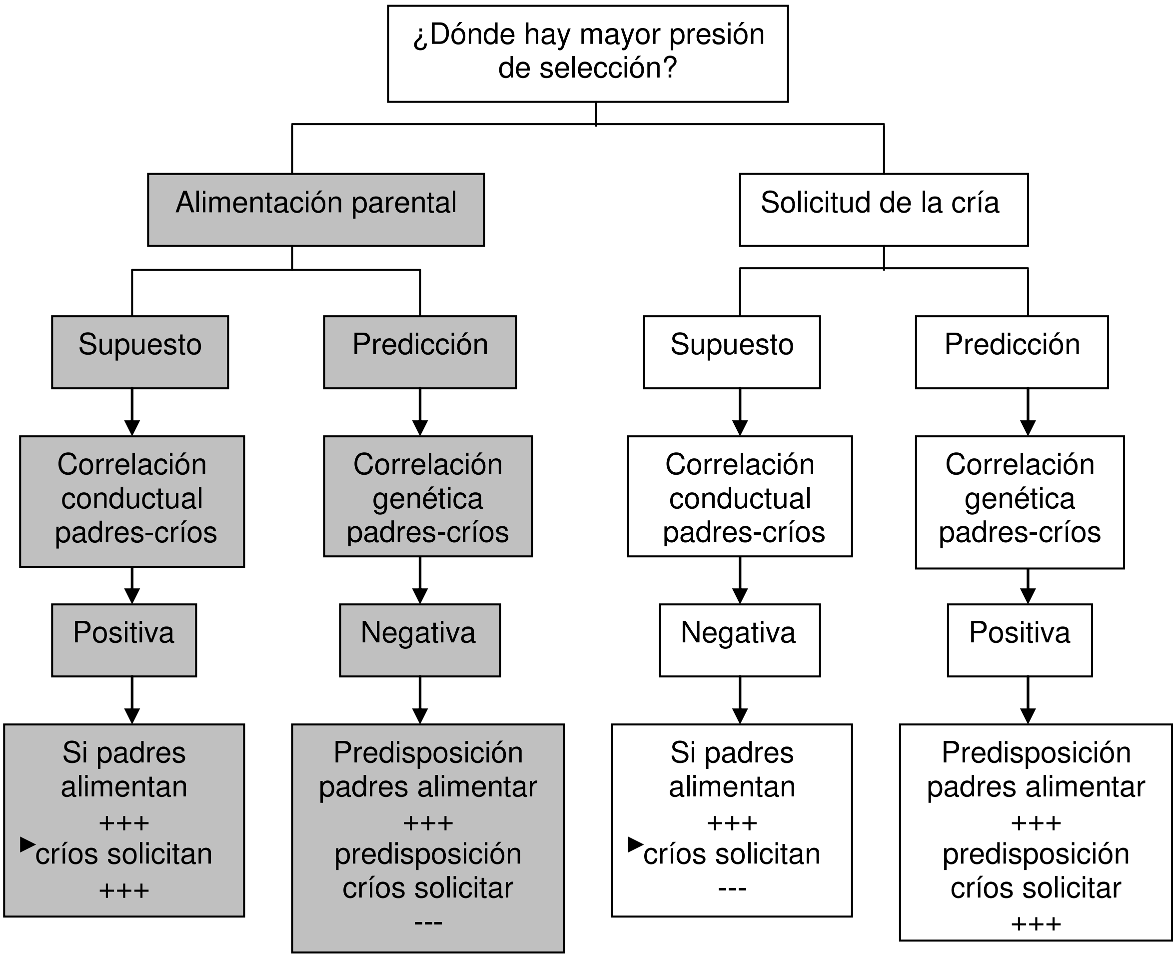

Figura 1. Predicciones del modelo de coadaptación madre-cría en función de la característica sobre la que hay mayor presión de selección (Kölliker *et al.* 2005). El modelo establece como pre-requisito para la selección de determinadas combinaciones madre-cría sobre otras la ocurrencia de una correlación conductual entre provisión parental y solicitud de la cría. El tipo particular de combinación favorecida por la selección depende de la dirección específica de la correlación conductual.

Las presiones de selección sobre los cuidados parentales en especies de larga vida con un número reducido de eventos reproductivos, en las que los padres enfrentan más fuertemente el compromiso entre invertir en la reproducción actual e invertir en supervivencia y reproducción futura, podrían favorecer la selección de una coadaptación negativa entre la entrega de cuidados y la tasa a la que estos son solicitados (Kölliker *et al.* 2005). En especies de aves con cuidados bi-parentales prolongados, madre, padre y cría podrían estar sujetos a presiones de selección sobre el nivel óptimo de cuidados, descontando la posible interacción entre la inversión de ambos padres, en estas especies podría esperarse una coadaptación madre-cría tanto como una padre-cría, en caso de que tanto padre como madre fueran sensibles a las demandas por parte de la cría.

El bobo café (*Sula leucogaster*)

El bobo café (*Sula leucogaster*) es un ave marina de larga vida, que habita en los trópicos y presenta un sistema de apareamiento monogámico social con baja ocurrencia de cópulas extra pareja (Nelson 1978; Gañan *et al.* 2014). Tanto machos como hembras de esta especie, participan en la incubación de la puesta, y en la alimentación de las crías que puede durar hasta 5 meses, tiempo en que el pollo alcanza independencia nutricional (Dorward 1962; Nelson 1978). Durante las alimentaciones, los padres transfieren porciones de pescado semidigerido directamente en la boca de las crías varias veces al día (Nelson 1978; Tershy y Croll 2000). Esta especie presenta fratricidio obligado, por este motivo, aunque la puesta modal es de dos huevos y con frecuencia los dos eclosionan, el pollo que eclosiona primero elimina al más joven durante los primeros días de vida, incluso cuando la disponibilidad de alimento es suficiente para proveer a las dos crías (Drummond *et al.* 2003; Osorno y Drummond 2003). Durante la incubación y las primeras semanas de la crianza se require la presencia permanente en el nido de al menos unos de los dos padres, debido a que los huevos y los pollos jóvenes (3-4 semanas después de la eclosión) son más vulnerables a la acción de depredadores como gaviotas argénteas (*Larus argentatus*) y cangrejos rojos terrestres (*Gecarcinus lateralis*, Nelson 1978; Tershy y Croll 2000). Considerando la importancia del esfuerzo paterno durante la incubación y la crianza, las hembras de esta especie, podrían resultar beneficiadas al elegir machos que durante el cortejo, exhiban señales honestas de su capacidad parental, e incluso estas señales podrían tener valor informativo después del establecimiento de la pareja.

En las colonias de bobo café del pacífico oriental, los machos y las hembras difieren en la coloración del plumaje de la cabeza y la piel expuesta en las patas, tarsos, y alrededor del pico y ojos. En machos, el plumaje de la parte superior de la cabeza es blanco y a medida que deciende sobre el cuello se va tornando café-grisáseo hasta terminar en café obscuro. En las hembras el plumaje de la cabeza y cuello es café obscuro (Nelson 1978; Fig. 2a). Durante la época de cortejo los machos presentan una coloración azul-verdosa en la piel alrededor del pico, mientras que, la coloración de las hembras en esta temporada es amarilla-verdosa. Durante los despliegues de cortejo machos y hembras de esta especie, usan el pico para realizar presentaciones ritualizadas del material de anidación, chocan sus picos por los extremos laterales de forma repetida y solamente los machos, caminan junto a la hembra girando la cabeza de lado a lado y apuntan hacia el cielo con el pico exponiedo la piel descubierta que se encuentra por debajo de este (Nelson 1978; Fig. 2a y b).

Propuesta

Considerando la posible existencia de un compromiso fisiológico entre inversión reproductiva y automantenimiento somático mediado por un desbalance oxidante, en el capítulo II se evaluó si la inversión reproductiva en ornamentos sexuales, puesta y esfuerzo parental genera costos oxidantes. Partiendo de la idea de que las coloraciones dependientes de carotenoides son indicadores honestos de la condición del individuo, y de la evidencia de la presencia de carotenoides en la piel del gular del bobo café (apendice I), en el capítulo III se evaluó la capacidad de la coloración del gular de los machos, duante el cortejo, para indicar beneficios directos e indirectos a las hembras. En el capítulo IV, se estudió si la coloración del gular de machos y hembras varía a lo largo del evento reproductivo, y si esta variacion es potencialmente perceptible por coespecíficos. Finalmente, en el capítulo V se evaluó la posible ocurrencia de una coadaptación entre las señales de solicitud de la cría y la disposición de los padres a proveer alimento.

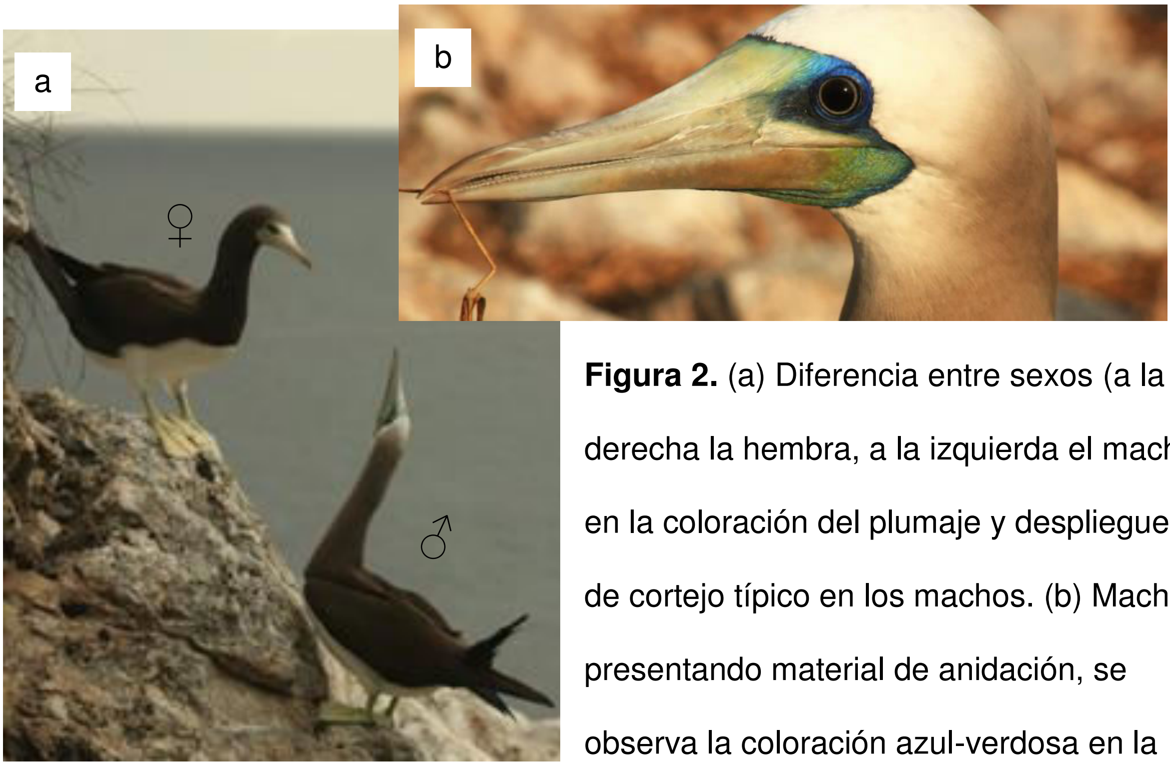

Figura 2. (a) Diferencia entre sexos (a la derecha la hembra, a la izquierda el macho) en la coloración del plumaje y despliegue de cortejo típico en los machos. (b) Macho presentando material de anidación, se observa la coloración azul-verdosa en la piel expuesta bajo el pico (gular) exhibida durante el cortejo. Fotos cortesía de Víctor Argaez.

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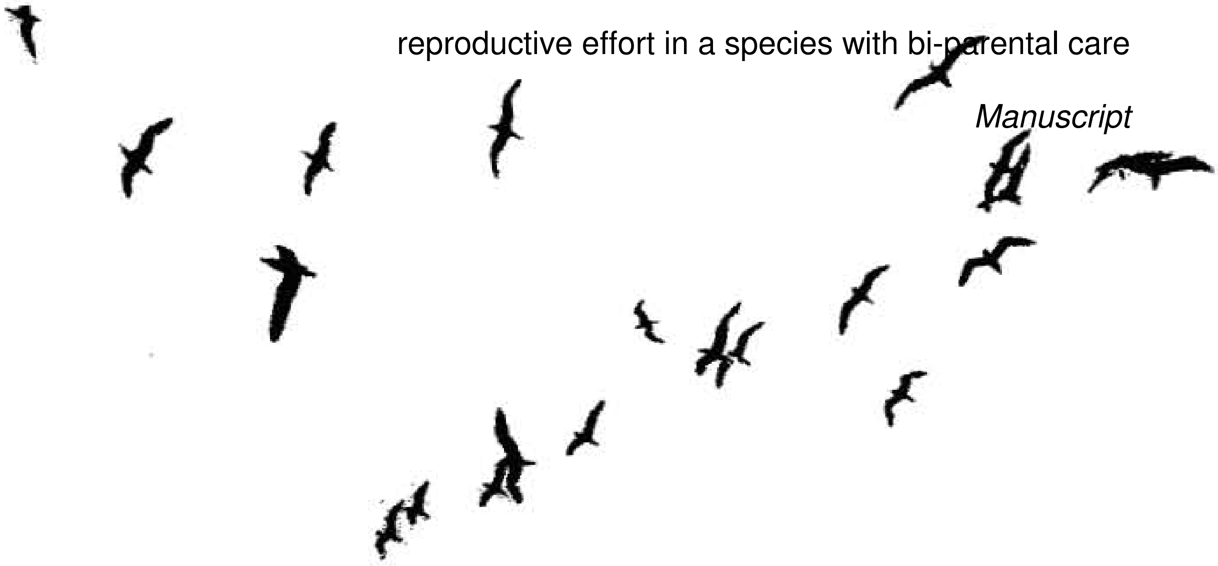
CAPÍTULO II

Oxidative stress during courtship affects differentially male and female reproductive effort in a species with bi-parental care

Manuscript



Oxidative stress during courtship affects differentially male and female reproductive effort in a species with biparental care

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Running title: Oxidative stress and reproductive effort by sex

Summary statement: Oxidative stress may arise as a cost from pre-laying reproductive investment in both sexes, and constrain egg and offspring investment of females

ABSTRACT

Whether oxidative stress modulates reproductive effort, either by arising as a cost or imposing a constraint is not clearly known yet. Studies testing this hypothesis have produced mixed results. We evaluated whether oxidative stress imposes a cost or a constraint on reproduction of a long-lived seabird with prolonged and obligate biparental care, the brown booby (*Sula leucogaster*). Additionally, using a cross-fostering experiment we explored genetic and environmental variation associated to oxidative status of offspring. At courtship, we found a positive association between skin color and lipid peroxidation (LP), no-association between color and ROS, and an increase in reactive oxygen species level (ROS) as laying approached in both sexes. Furthermore, in females, higher ROS at courtship was related to smaller first eggs laid and chicks with slower rate of increase in body mass, and higher LP was associated with shorter time of offspring attendance. No evidence for an oxidative cost of parental effort was found; male and female ROS levels decreased from courtship to parental care, whereas non-significant variation in LP levels was found. Finally, ROS levels of offspring were unrelated to rearing and genetic parents ROS at courtship, but LP levels were positively associated with rearing mother and father LP, and genetic father LP at courtship, suggesting LP might have an environmental and genetic component. Overall, results suggest that oxidative stress may play a different role during courtship and parental care in borwn booby males and females. Oxidative stress might arise as a cost from investment during courtship in both sexes, and constrain investment in eggs and offspring in females.

Key words: color, constrain, cost of reproduction, life-history trade-off, parental care.

The existence of a physiological trade-off based on limited resources has been traditionally proposed to explain the cost of reproduction, as resources invested in reproductive activities are no longer available to other life functions (Stearns, 1992; Reznick *et al.,* 2000). However, reproduction and their associated processes might also directly inflict somatic damage and affect other life-history components through their physiological by-products (Harshman and Zera, 2007). Yet, the mechanisms underlying such type of cost remain unclear (Rose and Bradley, 1998; Zera and Harshman, 2001; Barnes and Partridge, 2003). An increased susceptibility to oxidative stress, as by-product of an increase in energy used during reproduction, has been suggested to be a potential physiological short- and long-term cost of reproduction, independently of an energy or resource allocation trade-off (von Schantz *et al.,* 1999; Salmon *et al.,* 2001; Wang *et al.,* 2001, Alonso-Alvarez *et al.,* 2004; Monaghan *et al.,* 2009; Costantini, 2008).

Aerobic species use oxygen to fuel biological processes and, as by-product of the ATP production in the mitochondria, reactive oxygen species (ROS) are released. However, uncoupling allows mitochondria to reduce ROS releasing, by sacrificing ATP production (Speakman *et al.,* 2004; Speakman *et al.,* 2014). Oxidative stress occurs when the normal redox signaling processes of the cell are disrupted, leading to harmful changes in the antioxidant homeostasis (Jones, 2006). Although ROS have an important cellular signaling role (Hurd and Murphy, 2009; Dickinson and Chang, 2011), the un-neutralized ROS are involved in further reactions than can damage lipids (hereafter lipid peroxidation, LP), proteins and DNA (Beckman and Ames, 1998; Finkel and Holbrook, 2000). The increment in energy expenditure observed during the reproductive effort is associated with an increased basal metabolic rate (Angilletta and Sears, 2000; Nilsson, 2002; but also see Williams and

Vézina, 2001), potentially leading to ROS over-production, and consequently, temporarily augmented oxidative stress susceptibility (Bergeron *et al.,* 2011).

Evidence regarding the existence of a physiological cost of reproductive effort through an oxidative threat imposed by ROS over-production remains unclear. A group of studies using wild animal models find an association of reproduction with increased daily energy consumption and oxidative damage in lipids and proteins (Bergeron *et al.,* 2011; Heiss and Schoech, 2012; Fletcher *et al.,* 2013; Costantini *et al.,* 2014). Furthermore, studies in wild and captive animals present evidence for a potential oxidative imbalance resulting from reproduction, measured as higher production of reactive oxygen metabolites (Casagrande *et al.,* 2012; Stier *et al.,* 2012; Guindre-Parker *et al.*, 2013), decreased antioxidant capacity (Wiersma, 2004; Costantini *et al.,* 2010; van de Crommenacker *et al.,* 2011), and diminished resistance to oxidative burst (Alonso-Alvarez *et al.,* 2004; Bertrand *et al.,* 2006; Losdat *et al.,* 2011; Christe *et al.,* 2012). Nevertheless, no-relationship between reproduction and oxidative damage has also been reported (Nussey *et al.,* 2009; Isaksson *et al.,* 2011; Aloise-King *et al.,* 2013; Wegmann *et al.,* 2015). Interestingly, some studies have found better antioxidant response in females' liver cells during parental care (Yang *et al.,* 2013; Xu *et al.,* 2013), and even less oxidative damage in serum, liver, kidney and muscle cells of reproductive individuals (Garratt *et al.,* 2011; Ołdakowski *et al.,* 2012; Garratt *et al.,* 2013) compared to non-reproductive counterparts. Recently, the activation of a protective shielding triggered by the change from a non-reproductive status to a reproductive one has been proposed as a mechanism to prevent excessive oxidative damage resulting from parental investment, especially on mothers and offspring through a maternal effect (Blount *et al.,* 2015). However, the physiological pathway behind such mechanism has not been explicitly proposed yet. This shielding mechanism has been suggested to explain the apparently contradictory results on the field up to date.

An alternative, yet non-exclusive, explanation for the inconsistent results suggests a confounding influence of a potential constraining effect of oxidative stress on reproductive investment. This hypothesis states that the constraining effect of oxidative stress might prevent individuals from increasing their reproductive effort (Kaur *et al.*, 2006; Reglero *et al.,* 2009), when they are in an oxidative unbalance (Pike *et al.,* 2007), staying thereby safe from a potential oxidative damage in bio-molecules during reproduction (Metcalfe and Alonso-Alvarez, 2010; Stier *et al.,* 2012). Accordingly, studies evaluating the influence of oxidative stress on colorful sexual traits suggest that higher levels of oxidative stress indeed constrain the investment in such ornaments, particularly when they are carotenoid-dependent (Torres and Velando, 2007; Mougeot *et al.,* 2010; Alonso-Alvarez and Galván, 2011; Vergara *et al.,* 2012; but see Isaksson and Andersson, 2008). Additionally, supporting the idea of the constraining effect, lower resistance to oxidative burst (i.e. red blood cells resistance) and higher oxidative damage (i.e. lipid peroxidation and protein carbonilation) before pair formation, clutch establishment and egg hatching have been associated respectively with smaller litter size at birth (Stier *et al.,* 2012), lower male parental effort (Heiss and Schoech, 2012), smaller clutch size, lower hatching probability and lower survival probability of the parent (Bize *et al.,* 2008).Yet, no association between oxidative markers at arrival to the courting site and territory quality or number fledgings has been reported (Guindre-Parker *et al.,* 2013). However, the number of studies exploring the potential negative impact of oxidative stress on the investment in different reproductive stages, and the cumulative effect of an oxidative unbalance from one stage over the next ones is still reduced on wild species (Bize *et al.,* 2008; Petes *et al.,* 2008; Metcalfe and Alonso-Alvarez, 2010).

The brown booby (*Sula leucogaster*) is a long-lived species with an extended period of obligate biparental care. In this species, modal clutch size is two eggs and both parents incubate the clutch during 42 days on average. Brown boobies show obligate siblicide. During the first days of life the older chick typically eliminates the younger one, even when enough food is available for both chicks (Drummond *et al.,* 2003; Osorno and Drummond, 2003), resulting in only one offspring surviving per breeding event. The offspring is attended and fed by both parents during an approximate three month-period (Tershy and Croll, 2000). During this period, parents feed their chicks by transferring small portions of semi-digested fish directly into the chick's mouth (Nelson, 1978). Eggs and young chicks are vulnerable to predatory pressures; in consequence, they cannot be left unattended by the parents.

During courtship, males display green-bluish coloration on bare skin under the bill (hereafter gular color), and females exhibit yellow-greenish color in this tegument. Gular skin color in both sexes has been found to be carotenoid-dependent (B. Montoya, C. Flores and R. Torres, unpublished data), and in males this color indicates direct and indirect benefits to females (Montoya and Torres, 2015). Males with greener gulars attend the offspring longer, feed them more, and have chicks with higher body mass gain and structural growth, suggesting that gular color is a sexual signal that indicates male capacity to invest in parental duties (Montoya and Torres, 2015).

In this study, we performed a clutch cross-fostering experiment to: (i) deviate parents from the optimal investment as suggested by Metcalfe and Alonso-Alvarez (2010), hence, disrupting any potential parent-offspring co-adaptation over parental care (Kölliker *et al.*, 2005), and (ii) distinguish environmental from genetic effects on offspring measurements. To estimate oxidative stress, we measured at courtship levels of ROS production and lipid peroxidation (LP), which is a measurement of oxidative damage to lipids. The aims of this work were to evaluate whether oxidative stress (i) at courtship, modulates reproductive investment either by arising as a cost or imposing a constraint on reproductive effort, and (ii) in offspring, depends on rearing parents and/or genetic parents variation associated. First, if gular color in the brown booby is impacted by an oxidative unbalance, we expected a negative association of this colored ornament during courtship with the levels of ROS and LP. Second, if oxidative stress constrains reproduction, we predicted that males and females with higher levels of ROS or LP during courtship would have later laying dates, smaller clutch volume, lower offspring attendance, lower food provisioning and chicks with lower body mass and size. Third, if oxidative stress arises as a cost of reproduction, we expected that males and females would increase ROS and LP levels during parental care compared with their own courtship levels, and this increment would be positively associated with their breeding effort (i.e. offspring attendance, food provisioning and chick growth). Finally, if oxidative stress has environmental (rearing parents) variation associated, we expected offspring ROS and LP to be correlated with rearing parents ROS and LP, while if oxidative stress is associated with genetic variation (genetic parents), we expected a correlation between chick ROS and LP with genetic parents ROS and LP levels.

METHODS

The study was carried out at the brown booby breeding colony of Isla Larga, Parque Nacional Islas Marietas, Nayarit, Mexico (20º 41'N 105º 36' W) from June to September 2011. During the courtship period, we captured 52 pairs and individually marked both members of each pair with a white numbered polymethylmethacrylate leg band (PMMA leg bands; Interrex, Poland). For each captured bird body mass (± 20 g), ulna length (± 1 mm) and gular color were measured. Gular color was quantified as the mean reflectance curve of three sequential measurements of non-overlapping patches from the gular skin, roughly 1 cm apart, using a portable spectrophotometer that determines the reflectance from 360 to 740 (± 10) nm (MINOLTA CM 2600d, Osaka, Japan). To determine LP and ROS at courtship, a 3 mL blood sample was taken from the brachial vein of each bird. Blood samples were kept on ice until they were centrifuged at 10000 x g for 10 min, stored in the field during about two months in liquid nitrogen within a maximum of 2 hours from collection and maintained at -80°C until analyses were performed. Measurements and blood sampling of each bird were completed in roughly 10 minutes, pairs were handled simultaneously and then returned to their courting site. Pairs' courting sites were marked with a numbered flag and the study area was monitored daily between 18:00 and 20:00 h to determine laying date of focal pairs. On the day of laying, volume in mm^3 (length x $width^2$ x 0.51 / 1000, Hoyt 1979) of each egg was measured.

Cross-fostering

Fifteen days after the first egg of the clutch was laid, 52 clutches were randomly assigned ussing ballots to either the cross-fostered or the control group. In the cross-fostered group (n = 34, 28 two-egg and 6 one-egg clutches), complete clutches were swapped between pairs with similar laying date (± 3 days) and equal clutch size (1 or 2 eggs). To address another research question for a different experimental study, cross-fostered was made matching clutches with males that had the greatest difference in gular reflectance at 540 nm in the nests assigned to the cross-fostered group (peak wavelength in the green range, relationship of male color within swapped clutches $r_p = -0.50$ $P = 0.008$, see Montoya and Torres 2015). In the control group (n = 18, 12 two-egg and 6 one-egg clutches), the swapping procedure was simulated, but clutches were returned to their original nests (see Montoya and Torres 2015). Nests were monitored every 5 days during incubation, and daily after hatching until the chicks were 15 days old. Chick body mass (± 1 g) and, as a proxy of skeletal growth, ulna length (± 1 mm) were measured at the age of 1, 5, 10 and 15 days. When the chicks were 15 days old, chicks and parents were blood sampled (chicks 1 mL and parents 3 mL) to estimate levels of ROS and LP, and to determine the sex of the chicks according to Griffiths *et al.* (1998). All blood samples were processed as described above.

Throughout the experiment 13 nests were lost: no hatching occurred in 5 nests (3 cross-fostered and 2 control), and in 8 nests the surviving chick died before reaching 15 days of age (3 cross-fostered and 5 control). Hence, sample size for the analyses comprised 39 nests with a single chick that survived up to age of 15 days (36 first chicks and 3 second chicks hatched as sigletons), yet a potential design problem due to selective disappearance of the initial nests in the sample is not likely. Second chicks included in the sample, were not identified as extreme values in any of the analyses.

Behavioral records

Parental behavior and chick begging were recorded when the chicks were 1, 5, 10 and 15 (± 3 days) days old. Observations were performed from 07:00 - 09:00 h and from 17:00 - 21:00 h, the periods when most diurnal parental care activity occurs in this breeding colony (Montoya B unpublished data). Behavioral observations were carried out by four trained observers located at a distance of 3 - 6 m from the focal nest. Inter-observer reliability was above 90% at the beginning of the study and observers were uninformed about whether the observed nest belonged to the control or the cross-fostered group. Behaviors recorded were: (i) the absolute occurrence of provisioning by the male and the female (when a parent places the bill above the chick's head and the chick places its head into the parent's bill), (ii) the occurrence in 5 min intervals (1 - 0 records) of chick begging (when the chick raises the head and vocalizes with a "tac-tac" sound) to the male or the female, and (iii) the male and female offspring attendance (the minute and second of arrivals to and departures from the nest by each parent, including the times when the two parents overlapped at the nest site, Nelson 1978). For each chick, behaviors recorded during the four days of observation were pooled together for the analyses.

Protein quantification

Lipids require to be transported into the blood flow within lipoproteins, because they are hydrophobic (see Vance and Vance 2002). Hence, lipid peroxidation (LP) results from the attack to lipoproteins by ROS (see Halliwell and Gutteridge, 1999). Therefore, we quantified proteins to estimate the amount of available substrate into the blood flow for redox reactions, by doing so we aimed to avoid potential biases from estimating ROS and LP based only in the sample volume (Barja de Quiroga *et* *al*., 1991). We quantified the levels of plasma protein in each sample using the Bicinchoninic Acid (BCA) method for protein quantification. The protocol of the BCA protein assay kit (Pierce, Rockford, IL, USA) was followed adjusting the amount of sample from 20 µL to 10 µL. In this assay, the chelation of two molecules of BCA with one cuprous ion produces a purple colored reaction, which increases with protein concentration (Smith *et al.,* 1985). The absorbance of the resulting complex was measured at 562 nm of absorbance using an ELISA spectrophotometer (Bio-Rad Model 550). The results were calibrated with a standard curve of bovine albumin (Thermo Scientific 23225). To perform LP and ROS assays an approximate of 50 µg protein per sample is required, thus protein concentration in our plasma samples were 1:100 diluted before subsequent analysis to achieve the suggested concentration.

Lipid peroxidation (LP)

The thiobarbituric acid reactive substances (TBARS) assay estimates peroxidative damage to lipids through the formation of the pink chromogen $[TBA]_2$-malondialdehyde adduct (Halliwell and Chirico, 1993). Briefly, a 100 µL aliquot of the diluted plasma was added to 100 µL of trichloroacetic acid (10% v/v) and centrifuged at 3000 x g for 10 min. The supernatant was added to 1 mL of thiobarbituric acid reagent (0.375% TBA and 2% acetic acid), and the mixture was incubated at 92º C for 45 min. After reaction, samples were placed on ice for 10 min. The absorbance of thiobarbituric acid – MDA complex was measured at 532 nm of absorbance using an ELISA spectrophotometer (Bio-Rad Model 550). Estimates of lipid peroxidation were calculated as MDA equivalents interpolating into a concentration curve of 1,1,3,3-tetraethoxypropane (Fluka Chemie Co., USA) ranging from 0 to 5 nM. Throughout the document, lipid peroxidation is expressed as nmol MDA/mg protein. The average coefficient of variation (CV), measured as the ratio of the standard deviation to the mean and expressed as percentage, between replicates was lower than 5.11%.

Reactive oxygen species (ROS)

This technique is based on the oxidation of the non-fluorescent molecule dihydrorhodamine-123 to the fluorescent rhodamine-123 by H_2O_2 in presence of peroxidases to estimate reactive oxygen species production (ROS)(Henderson and Chappell, 1993). Briefly, 180 µL buffer A (140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM HEPES) and 20 µL dihydrorhodamine-123 (1 mM, Aldrich Chemical Co., Milwaukee, WI, USA) were added to 20 µL aliquots of the diluted plasma samples. The mixture was placed in a 96-well plate and read at 505 nm in an ELISA spectrophotometer (Bio-Rad Model 550). The results were interpolated from a standard curve of rhodamine-123 (Aldrich Chemical Co., Milwaukee, WI, USA) in buffer A ranging from 0 - 10 µM. Throughout the document, ROS quantity was expressed as µmol rhodamine-123 /mg protein. The average CV between replicates was lower than 4.54%.

Statistical analysis

Male gular color variation was synthesized by performing a Principal Component Analysis (PCA) that included UV (sum of reflectance from 360 – 400 nm / sum of total reflectance from 360 - 740 nm), blue (sum of reflectance from 430 - 470 nm / total reflectance) and green chroma (sum of reflectance from 480 - 550 nm/ total reflectance). Three PCs were extracted explaining 59.86%, 34.38% and 5.74% of the variation (eigenvalues PC1 = 1.79, PC2 = 1.03 and PC3 = 0.17). For the statistical analyses, we used PC1 (hereafter, male gular color) with factor loadings of -0.60 for green chroma, 0.71 for UV chroma, and 0.34 for blue chroma. From females' gular color, we calculated maximum reflectance value within the 360 - 740 nm (hereafter, female gular color). Courtship color measurements of 6 females were missed in the field, 4 of those 6 females were recaptured at parental care, then the color at that period was used as an estimate of color at courtship (female gular rmax at courtship and at parental care did not differ, paired *t*-test = -87, $P = 0.38$, $n = 29$).

Prior to statistical analyses, ROS and LP estimates were natural-log transformed. Independent models were fitted to explore separately the effect of ROS and LP on each response variable (i.e. gular color, laying date, egg volume, parental provisioning, chick attendance, rate of offspring size and body mass increase, offspring ROS and LP). General linear models with normal error distribution were fitted to evaluate the effects of ROS and LP on all the response variables except for parental provisioning. To analyze parental provisioning generalized linear models with negative binomial distribution to correct for over-dispersion were fitted. ROS estimates of 4/39 males, 14/39 females and 4/39 chicks, and LP estimates of 9/39 males, 10/39 females and 3/39 chicks could not be obtained due to samples were missed during field work, handling or laboratory analyses. Consequently, sample size varies among analyses, and the number of covariates in statistical models was maintained in the minimum to deal with an unbalanced data set. Minimal adequate models were obtained by backward deletion procedure of non-significant terms ($\alpha = 0.05$). Effect size for principal results was calculated through eta-squared using the package "lsr" (Navarro, 2015), and results were included in the figure legend or in the main text (when no-figure available). Residuals from all final models were normally distributed. Analyses were carried out using R 3.0.2 (R core team, 2014) or SAS software 9.0 (SAS institute, 1999).

All models included ROS or LP levels of rearing parents as main variables, and models of reproductive effort during the chick-rearing period included the swapping treatment and its interaction with the parents' markers of oxidative stress. Additionally, models to analyze the effects of oxidative markers on gular color included days to laying as a covariate, and its interaction with the oxidative markers. In the model of male gular color an outlier was identified and dropped (Id 21, outlier test $P = 0.01$). Models to evaluate the potential constraining effect of ROS and LP on laying date, clutch volume and first egg volume included LP or ROS of both parents as main variable and the interaction between mother and father oxidative markers. As ROS levels resulted significantly correlated with days to laying ($r_p = -0.40$, $P < 0.001$), the residuals of this correlation were used in further analyses. Models to analyze the effect of rearing parents ROS or LP levels on mass and size at hatching, chick mass gain rate, and chick ulna growth until 15 days, included rearing parents' oxidative marker as main variable, egg volume as a covariate and the interactions of rearing parents' oxidative markers with the swapping treatment. Only in the analyses of hatchling mass and chick mass gain rate, ulna length at hatching and ulna growth rate were respectively included to control for structural size. Effects of oxidative stress at courtship on parental feeding and offspring attendance included the swapping treatment as a fixed factor, chick body mass at hatching as covariate and the interaction between the swapping treatment and parental oxidative marker. In the analyses of parental provisioning, chick begging rate and its interaction with parental oxidative markers were included.

To evaluate the potential occurrence of an oxidative cost of reproduction, we fitted general linear models of repeated measures to analyze the change in rearing parents ROS and LP levels from courtship to parental care. Models included nest identity as subject term, reproductive stage (courtship or parental care) as the repeated measure, swapping treatment as fixed factor, and chick body mass at 15 days, parental provisioning and nest attendance as covariates, and the interactions of swapping treatment with parental provisioning and offspring attendance.

Finally, the effect of genetic and environmental variation on offspring ROS and LP levels was not evaluated within the same model due to lack of statistical power associated with missing values. Therefore, to explore this question, we fitted independent models including either the genetic mother and father ROS and LP levels, or the rearing mother and father ROS and LP levels as main variables. Both models included swapping treatment as a factor, egg volume, rate of body mass gain, chick weighed begging (sum of chick begging rate to mother and father plus one divided by sum of provisioning of father and mother plus one, one was added to avoid zeros in the numerator or denominator) as covariates, and the interactions between the parents oxidative markers with the swapping treatment.

RESULTS

During courtship, ROS levels of males and females increased as laying approached (males, $F_{1,37} = 8.16$, $P = 0.007$, $\beta = -0.02 \pm 0.008$, eta-squared = 0.18; females $F_{1,26} = 4.24$, $P = 0.050$, $\beta = -0.02 \pm 0.01$, eta-squared = 0.14); yet LP levels were unrelated to laying date (males, $F_{1,30} = 0.07$, $P = 0.79$; females $F_{1,30} = 0.55$, $P = 0.46$). After controlling for variation related to the timing of laying, ROS production and LP levels were positively correlated during courtship for males (ROS $F_{1,29} = 4.07$, $P = 0.053$, days to laying $F_{1,29} = 1.23$, $P = 0.27$), but not for females (ROS $F_{1,25} = 0.01$, $P = 0.91$, days to laying $F_{1,25} = 0.12$, $P = 0.73$). ROS and LP at courtship within pairs were not associated (LP $F_{1,24} = 0.38$, $P = 0.54$, ROS $F_{1,25} = 2.29$, $P = 0.14$).

Are sexual ornaments related to oxidative stress level?

During courtship, gular color was not associated with ROS levels of males ($F_{1,37} = 0.009$, $P = 0.92$; days to lay $F_{1,36} = 0.52$, $P = 0.47$; ROS x days to laying $F_{1,35} = 0.21$, $P = 0.65$) or females ($F_{1,25} = 2.02$, $P = 0.17$; days to laying $F_{1,24} = 1.46$, $P = 0.24$; ROS x days to laying $F_{1,23} = 3.94$, $P = 0.06$). However, gular color was positively related to the level of LP of males and females. Males with greener gular had greater LP levels as laying date approached (LP $F_{1,27} = 8.05$, $P = 0.009$; days to laying $F_{1,27} = 6.41$, $P = 0.02$; LP x days to laying, $F_{1,27} = 6.73$, $P = 0.02$; Figure 1a). Females with brighter yellow gular had greater LP levels ($F_{1,29} = 6.43$, $P = 0.02$; Figure 1b), regardless of the timing of laying (LP x days to laying $F_{1,27} = 0.32$, $P = 0.58$).

Does oxidative stress during courtship constrain reproductive effort?

Laying date and egg size

Males and females with higher ROS levels during courtship established earlier clutches (male ROS $F_{1,24} = 6.32$, $P = 0.02$, eta-squared = 0.14; female ROS $F_{1,24} = 5.62$, $P = 0.03$, eta-squared = 0.12; interaction $F_{1,23} = 0.19$, $P = 0.66$). Total clutch volume was not associated with male or female ROS (male ROS $F_{1,21} = 0.41$, $P = 0.53$, female ROS $F_{1,22} = 1.37$, $P = 0.25$, interaction $F_{1,20} = 0.58$, $P = 0.45$). However, females with higher ROS laid smaller first eggs ($F_{1,24}$ = 6.94, P = 0.01, eta-squared = 0.22), while male ROS was not associated with first egg volume (male $F_{1,23}$ = 0.03, P = 0.86, male x female $F_{1,22}$ = 0.07, P = 0.79). Within pairs, LP levels were not related to laying date (male LP $F_{1,30}$ = 2.30, P = 0.14, female LP $F_{1,23}$ = 0.16, P = 0.69, interaction $F_{1,22}$ = 0.38, P = 0.54), clutch volume (male LP $F_{1,26}$ = 0.79, P = 0.38, female LP $F_{1,20}$ = 0.001, P = 0.97, interaction $F_{1,19}$ = 0.01, P = 0.91), or first egg volume (male LP $F_{1,29}$ = 0.51, P = 0.48, female LP $F_{1,22}$= 0.03, P = 0.86, interaction $F_{1,21}$ = 0.58, P = 0.45).

Chick growth

Rearing mothers with higher ROS levels during courtship had chicks with lower rate of body mass gain (β = -3.46 ± 1.61, Table 1, Figure 2a). Rearing father ROS level was not associated with chick rate of increase in body mass (Table 1). Rearing mother and father ROS were not associated with hatchling body mass, ulna length, or chick structural growth (Table 1). Rearing parents' LP levels were unrelated to hatchling body mass and size, or the rates of chick body mass gain and structural growth (Table 1). Swapping treatment or its interaction with parental ROS and LP were not related with chick growth (Table 1).

Parental investment

Rearing parents ROS during courtship was not associated with mother or father provisioning or chick attendance (Table 2). However, mothers with higher LP levels spent less time attending their chicks (β = -4.02 ± 1.78, Table 2, Figure 2b), yet maternal LP was not associated with female provisioning effort (Table 2). Father's LP was unrelated with chick attendance or provisioning (Table 2). Swapping treatment or its interaction with parental ROS and LP were not related to mother or father parental investment (Table 2).

Is oxidative stress a cost of reproductive effort?

Parental oxidative stress change after parental care

Males and females had higher ROS levels during courtship than during parental care (Table 3, Figure 3). Chick mass at 15 days, parental provisioning, chick attendance, swapping treatment or its interaction with parental provisioning or attendance were all unrelated with the change in ROS from courtship to parental care showed by parents (Table 3). Male and female LP levels did not significantly vary from courtship to parental care and were not related to any of the covariates tested (Table 4).

Are oxidative stress markers in offspring associated with rearing and/or genetic parents' oxidative markers?

Chick oxidative stress

Chick ROS levels were unrelated to rearing or genetic parents ROS levels (Table 4). The swapping treatment, its interaction with rearing parents ROS, and the covariates included in the analyses were not associated with chick ROS levels (Table 4). However, chick LP levels were positively related to rearing mother LP ($\beta = 0.30 \pm 0.15$, Figure 4a) and rearing father LP levels ($\beta = 0.78 \pm 0.23$, Table 4, Figure 4b). Chicks from control group had higher LP than chicks from swapped nests, but the interaction between swapping treatment and rearing parents LP was not significant (Table 4). Additionally, chicks from larger eggs showed higher LP levels ($\beta = 0.02 \pm$

0.01, Table 4). Interestingly, chick LP was associated with genetic father LP ($\beta = 0.86 \pm 0.28$, Table 4, Figure 4c), but not with genetic mother LP levels (Table 4). Rate of increase in body mass and chick weighed begging were not related to LP levels of chicks (Table 4).

DISCUSSION

The proposal that oxidative stress plays a role as mediator of reproductive investment has motivated numerous studies (Reviewed in Blount *et al.,* 2015; Speakman *et al.,* 2015). However, evidence of such effect is still mixed. In this study, we evaluated whether oxidative stress constrains reproductive effort or results as a proximate cost of reproductive investment, and whether there is genetic and/or environmental variation associated with oxidative status in offspring. We found that oxidative stress (i) during courtship, might be a cost of displaying carotenoid-dependent colorful gulars and establishing earlier clutches, but not of parental care effort, (ii) during courtship, may constrain female reproductive investment in eggs and offspring, but not male investment during the chick rearing period, and (iii) in offspring, might result from genetic and environmental variation. Eta-squared for these results variated between 0.10 and 0.23, indicating medium to large effect sizes (Cohen, 1998)

In males, ROS levels were higher when laying date approached (effect size = 0.18), and lipid damage was higher when males displayed greener gulars closer to the laying date (effect size = 0.19). Additionally, ROS and LP levels were positively associated. These results suggest that developing colorful ornaments might entail an oxidative cost. Furthermore, they point that reproductive investment in ornaments and courtship, in general, might increase as laying date approaches. In the brown booby, male skin color is associated with direct and indirect benefits for females (Montoya and Torres, 2015). Hence, male gular color likely functions as a sexual trait used by females during mate choice. Therefore, enhancing gular color near to the laying date may stimulate female preferences conferring reproductive benefices to more ornamented males. The role of male skin color as a signal during courtship might be maintained through a handicap mechanism, where individuals than can afford temporary higher levels of oxidative damage, can also invest heavily in colorful ornaments, because they are in better general condition (Zahavi, 1975; Grafen, 1990; Iwasa *et al*., 1991; Alonso-Alvarez *et al*., 2007). Interestingly, in females, ROS levels were also higher as laying date approached and brighter gulars were associated with higher levels of lipid peroxidation (effect size = 0.14 and 0.18 respectively). In the brown booby, mutual mate choice is expected because of the long period of biparental care (Burley, 1977; Amundsen, 2000; Kokko and Johnstone, 2002), and if mutual mate choice occurs assortative mating may arise (Johnstone *et al.,* 1996). Thus, females might pay an oxidative cost for displaying brighter gulars to attract colorful males, obtaining the direct and indirect benefits that greener males offer (Montoya and Torres, 2015). Accordingly, we recently found that in the brown booby there is assortative mating by color (Montoya, B. and Torres, R. unpublished data). Our results suggest that, in both sexes, early reproductive investment, such as developing colorful ornaments entails an oxidative cost (i.e. lipid damage), and getting prepared to clutch establishment, as probably more vigorous courtship or territory defense may increase individuals vulnerability to suffer oxidative stress (i.e. higher ROS production).

Does oxidative stress constraint reproduction?

A constraining effect of oxidative stress in reproductive investment is expected when individuals with higher oxidative damage cannot pay the cost for a high reproductive effort (Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010). Contrary to expectations, pairs with higher ROS levels established earlier clutches (effect size = 0.12 and 0.14 females and males respectively). Therefore, ROS levels do not appear to constrain earlier clutch establishment. However, as predicted when oxidative stress constrains reproductive investment, females with higher ROS laid smaller first eggs (effect size = 0.22). Hence, ROS levels may arise as a cost of earlier egg laying and constrain egg volume investment. This double role of ROS as potential cost and limit, suggests that females likely compromise investment in earlier laying dates against egg size. This suggested trade-off may benefit females by avoiding the breeding success decline suffered by late breeders (D’Alba and Torres, 2007; Verhulst and Nilsson, 2008). Additionally, in females higher ROS at courtship was related to lower rate of body mass gain in offspring and, higher lipid damage to shorter time of offspring attendance (effect size = 0.16 and 0.17 respectively). Hence, ROS levels and oxidative damage in lipids during courtship may constrain female reproductive investment in the egg and the offspring, but not male parental effort. These results indicate the existence of differences between sexes in the susceptibility to the reproductive effort, or in the strategies to cope with the reproductive demands. Future studies are needed to further explore such differences and evaluate the fitness impact of the decline in female reproductive investment due to higher ROS or LP levels.

Is oxidative stress a cost of reproduction?

An increment in the basal metabolic rate has been reported to occur during reproduction (Angilletta and Sears, 2000; Nilsson, 2002; but also see Williams and Vézina, 2001). The rise in energy demand stimulates ATP production, and as consequence, might increase the release of reactive species. Depending on the current oxidative balance of the individual, an augmented release of reactive species may eventually lead to damage in bio-molecules (Halliwell and Gutteridge, 1999). Contrary to our predictions, males and females showed lower ROS production during parental care in comparison with their own ROS levels during courtship, and no significant change in the level of lipid peroxidation between stages was found. Additionally, there was no association between the magnitude of the change in ROS or lipid peroxidation and our estimates of parental effort post-hatching. A feasible explanation for such results is that in the brown booby, breeding activities during courtship entail higher costs, as discussed above, than parental care during the first two weeks post-hatching. In long-lived species with limited breeding opportunities, such as the brown booby, selection is expected to favor breeding strategies that minimize the physiological costs of current reproduction and increase survival and future reproduction (Minchella and Loverde, 1981; Clutton-Brock, 1984; Erikstad *et al.*, 1998; Kirkwood and Austad, 2000). Thereby, a constraining effect of oxidative stress on reproductive investment is more likely to occur in these species, than an oxidative cost, as was found in this study and was previously reported in other species with similar life-history characteristics (*Ovis aries*, Nussey, 2009; *Pygoscelis adeliae*, Beaulieu *et al.*, 2011). Alternatively, the variation found in ROS levels and the non-significant change in oxidative damage between courtship and parental care, could respond to environmental changes (i.e. precipitation level, ocean and ambient temperature, prey availability) rather than to the reproductive effort (Metcalfe and Monaghan, 2013). The relative reproductive synchrony of the brown booby breeding colony where this study was carried out could have favored a confusing effect. Disentangle the effect of these confounding variables in the wild is challenging, because measuring pairs out of the colony breeding peak would include additional individual variation associated with the optimal breeding time under those environmental conditions, but needs to be addressed.

Are oxidative stress markers in offspring associated with rearing and/or genetic parents' oxidative markers?

Until some years ago, the role of genetic and rearing parents' variation in oxidative stress in early-life had rarely been assessed (Metcalfe and Alonso-Alvarez, 2010). In our study, offspring ROS levels were not associated with either rearing or genetic parents ROS levels. Considering that our ROS measurement is sensitive mainly to H_2O_2 production (Henderson and Chappell, 1993) and this species are only one type of several kinds ROS that can be released, we could underestimate ROS variation and thus failed to detect any environmental or genetic influence. Alternatively, there could be not genetic variation related to the rate of the electron transport chain and its associated ROS production, and genetic variation reported in other studies, might be associated with ROS production in response to challenging arrangements rather than baseline levels (Olsson *et al.*, 2009). Offspring lipid peroxidation was positively associated with rearing mother and father lipid peroxidation, suggesting an environmental component behind the oxidative damage to lipids. The functioning of the antioxidant barrier shown to be more environmentally dependent (rearing parents variation) in other studies (Costantini and Dell'Omo, 2006; Norte *et al.*, 2009; Olsson *et al*., 2009; but see Kim *et al.,* 2010) might relate to this result. We also found an association between genetic father and chick lipid peroxidation. This last result is similar to those in previous studies showing ROMs variation to have a genetic component (Costantini and Dell'Omo, 2006). ROMs (reactive oxygen metabolites) levels are a measurement of plasma oxidative metabolites and have been proposed to result from early macromolecules oxidative damage rather than from only ROS production (Costantini *et al*., 2007; Costantini *et al*., 2011; Costantini and Dell'Omo, 2006, 2015). The effect size of genetic father lipid peroxidation was larger (0.23), than the effect size of rearing parents' lipid peroxidation (0.10 and 0.19 female and male respectively) suggesting that variation in offspring lipid peroxidation could be more associated with genetic parents factors than with rearing fathers'. Unfortunately, due to our small sample size, we were unable to disentagle the relative role of environmental and genetic variation on offspring damage to lipids.

Conclusion

We found no evidence about an oxidative cost of reproduction estimated as an increment in oxidative damage to bio-molecules during parental care compared to courtship, or as an augmented oxidative damage resulting from higher parental effort. Interestingly, results suggest that lipid damage may arise from a stronger investment in sexual ornaments, and elevated ROS production from an early clutch establishment. Particularly in females, ROS levels might constrain investment in egg volume and offspring mass, whereas lipid damage might limit time of offspring attendance. The fact that we found a constraining effect of oxidative stress only on female reproductive investment suggests differences between sexes in the susceptibility to reproductive effort, or in the strategies to cope with the demands linked to it (Wiersma *et al.,* 2004; Bize *et al.,* 2008).

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

RT and BM designed the experiment. BM collected behavioral data and performed blood sampling. BM, ER and MV performed oxidative stress measurements and contribute during results discussion. BM and RT analyzed data and wrote the first draft of the manuscript. All authors contributed to later versions of the manuscript.

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FIGURES

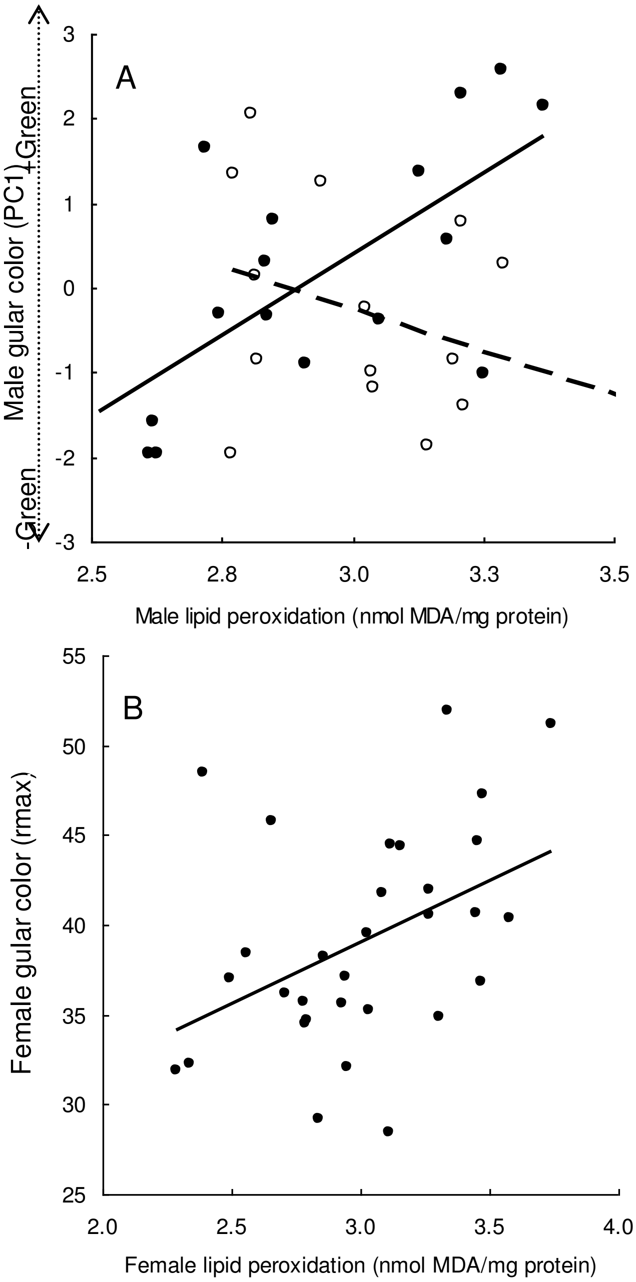

Figure 1. Gular color at courtship and lipid peroxidation. (A) Male gular color (PC1) and lipid peroxidation at courtship, as a function of the laying proximity. For clarity, only in the plot, PC1 was multiplied by -1, positive values indicate higher green chroma, and negative ones higher UV chroma. Closed circles correspond to measurements until 10 days before laying and open circles more than 10 days before laying. Eta-squared = 0.19. (B) Female gular color, measured as maximum reflectance, and lipid peroxidation at courtship. Eta-squared = 0.18.

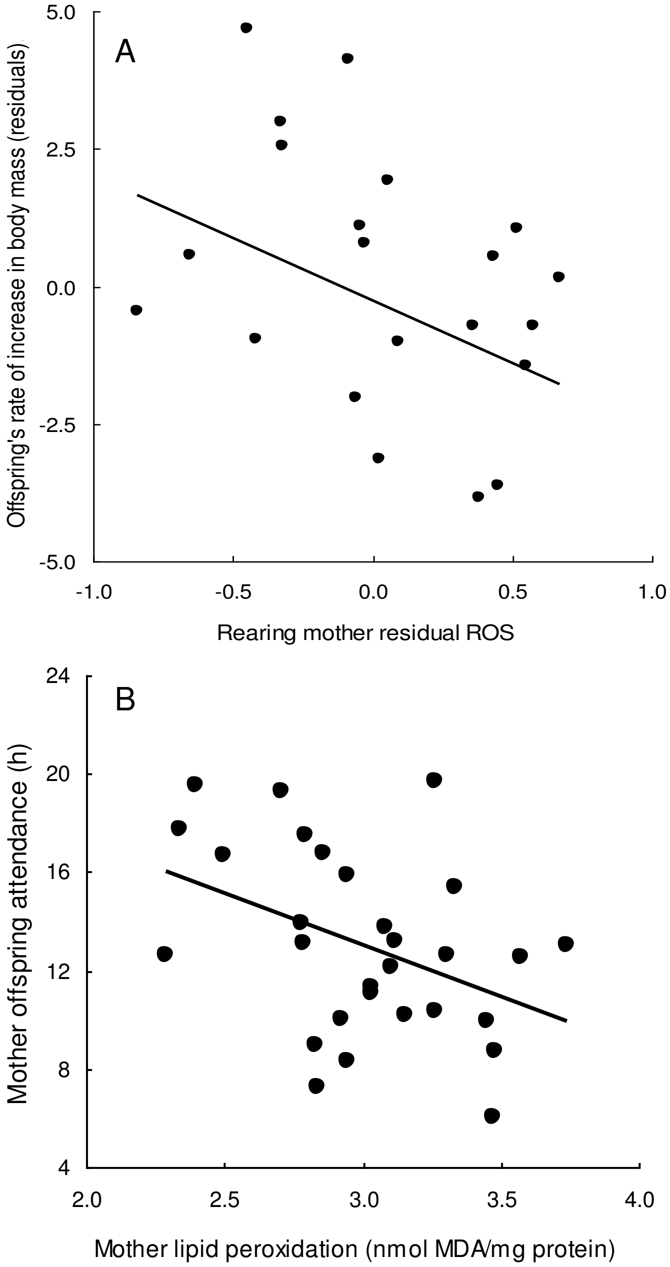

Figure 2. Maternal effort and oxidative stress. (A) Offspring's rate of increase in body mass from hatching to 15 days old and rearing mother residual reactive oxygen species (ROS) at courtship. Residual ROS was calculated as the residuals of the regression of mother ROS µmol/mg at courtship on days to laying. Residuals of the final model dropping mother residual ROS are plotted in the y axes. Eta-squared = 0.16. (B) Mother offspring attendance and its own levels of lipid peroxidation at courtship. Eta-squared = 0.17.

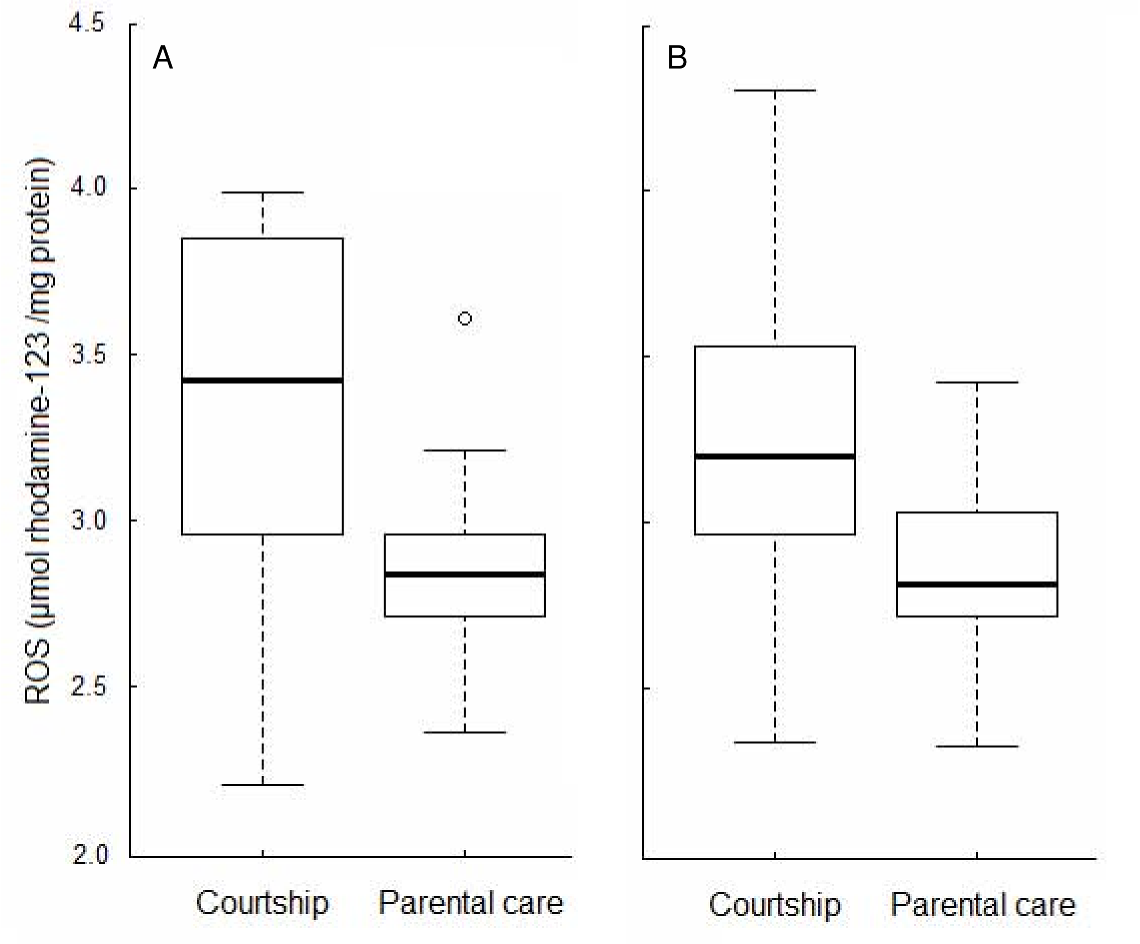

Figure 3. Reactive oxygen species level (ROS) at courtship and parental care in (A) females and (B) males. Measurements of ROS at parental care were obtained 15 days after the chick hatched. Boxes represent median (bold line), first quartile (lower limit) and third quartile (upper limit), whiskers correspond to maximum and minimum values excluding outliers. The open circle represents an extreme value.

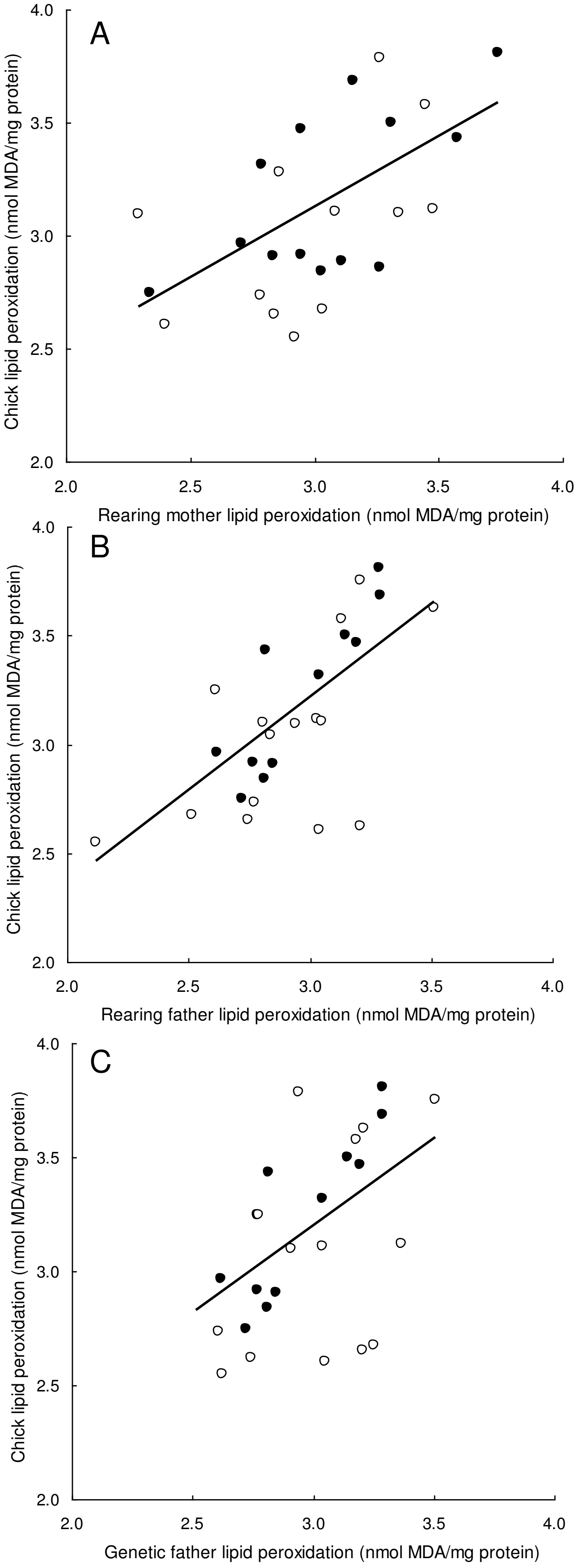
A
Chick lipid peroxidation (nmol MDA/mg protein)
Rearing mother lipid peroxidation (nmol MDA/mg protein)
B
Chick lipid peroxidation (nmol MDA/mg protein)
Rearing father lipid peroxidation (nmol MDA/mg protein)
C
Chick lipid peroxidation (nmol MDA/mg protein)
Genetic father lipid peroxidation (nmol MDA/mg protein)
2.0
2.5
3.0
3.5
4.0

Figure 4. Chick lipid peroxidation (LP) and parental lipid peroxidation (LP). Chick LP at 15 days post-hatched and LP at courtship of (A) rearing mother, (B) rearing father and, (c) genetic father. Closed dots represent control nests, and open dots to cross-fostered nests. Eta-squared = 0.10, 0.19 and 0.23 respectively.

TABLES

Table 1. Relationship of rearing parents reactive oxygen species (ROS) and lipidperoxidation (LP) levels at courtship with offspring mass and size.

	Hatching				Rate of change to 15d			
A. Rearing parents	**Body mass**		**Ulna**		**Body mass**		**Ulna**	
ROS	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Swapping treatment	1.12	0.30	0.13	0.72	1.88	0.18	0.22	0.64
Egg volume	**5.79**	**0.02**	0.001	0.97	**5.69**	**0.03**	0.26	0.61
Hatchling ulna	**19.97**	**<0.001**	-	-	-	-	-	-
Ulna growth rate	-	-	-	-	**22.32**	**<0.001**	-	-
Father ROS	0.04	0.85	0.04	0.85	0.15	0.70	0.28	0.60
Mother ROS	0.28	0.60	0.46	0.51	**4.64**	**0.04**	0.27	0.60
Father ROS x swapping	1.83	0.20	1.26	0.28	1.22	0.28	0.04	0.84
Mother ROS x swapping	1.71	0.21	0.06	0.81	0.06	0.80	0.01	0.90
Error df final model	34		37		19		35	

B. Rearing parents	**Body mass**		**Size**		**Body mass**		**Size**	
LP	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Swapping treatment	1.12	0.29	0.11	0.75	2.28	0.14	0.003	0.95
Egg volume	**5.79**	**0.02**	0.20	0.66	2.74	0.12	0.17	0.68
Hatchling ulna	**19.97**	**<0.001**	-	-	-	-	-	-
Ulna growth rate	-	-	-	-	**24.99**	**<0.001**	-	-
Father LP	0.17	0.68	2.68	0.12	0.07	0.79	2.25	0.14
Mother LP	0.16	0.69	0.41	0.53	0.04	0.84	1.07	0.21
Father LP x swapping	0.04	0.85	0.01	0.92	0.001	0.97	0.03	0.87
Mother LP x swapping	2.11	0.16	0.45	0.51	0.04	0.84	0.91	0.35
Error df final model	34		37		35		35	

Linear models showing the effect of rearing parents (A) reactive oxygen species (ROS; the residuals of the regression of ROS on days to laying were used for the analyses) and (B) lipid peroxidation (LP), on chicks' ulna length and body mass at hatching, and rate of change in these measurements to 15 days post hatching. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion and in bold, variables in the final model.

Table 2. Relationship of parents reactive oxygen species (ROS) and lipid peroxidation (LP) levels at courtship with further parental effort.

	Paternal effort				**Maternal effort**			
A. Parents levels of ROS	**Provisioning**		**Attendance**		**Provisioning**		**Attendance**	
	X^2	*P*	*F*	*P*	X^2	*P*	*F*	*P*
Swapping treatment	0.001	0.98	1.68	0.20	0.14	0.71	0.003	0.98
Hatchling mass	0.93	0.33	2.09	0.16	**12.63**	**<0.001**	0.002	0.98
Begging rate	**6.88**	**0.009**	-	-	**15.06**	**<0.001**	-	-
Father ROS	0.15	0.69	0.77	0.39	0.93	0.33	1.89	0.19
Mother ROS	0.001	0.97	0.02	0.89	0.98	0.32	0.05	0.82
Parent* ROS x swapping	0.10	0.76	0.58	0.45	0.19	0.66	0.002	0.96
Parent* ROS x begging	0.86	0.35	-	-	0.78	0.38	-	-
Error df final model	36		38		34		38	
B. Parents levels of LP	**Provisioning**		**Attendance**		**Provisioning**		**Attendance**	
	X^2	*P*	*F*	*P*	X^2	*P*	*F*	*P*
Swapping treatment	3.15	0.08	1.68	0.20	0.15	0.70	0.03	0.87
Hatchling mass	0.31	0.57	2.09	0.16	**12.63**	**<0.001**	0.25	0.62
Begging rate(*)	**6.88**	**0.009**	-	-	**15.06**	**<0.001**	-	-
LP father	0.73	0.39	0.48	0.50	0.04	0.85	2.08	0.16
LP mother	3.20	0.07	0.12	0.74	0.14	0.71	**5.63**	**0.03**
LP parent(*) x swapping	3.19	0.07	0.01	0.92	0.001	0.97	0.05	0.83
LP parent(*) x begging	2.09	0.15	-	-	0.001	0.98	-	-
Error df final model	36		38		34		27	

Linear models showing the effects of rearing parents (A) reactive oxygen species (ROS; the residuals of the regression of ROS on days to laying were used for the analyses) and (B) lipid peroxidation (LP), on offspring attendance time and provisioning. General linear models with normal error distribution were use to analyze attendance time, while generalized linear models with Poisson error distribution were used to analyze provisioning. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion and in bold, variables in the final model

(*) Models evaluating the effects of LP of ROS on *paternal* provisioning or attendance, included only the begging rate to the *father* and the interaction between *father* LP or ROS with the swapping treatment or the chick begging rate to the *father*, begging rate to the mother, or the interaction between mother LP or ROS with other variables were not included. The equivalent procedure was followed when fitting the models evaluating the effects of LP of ROS on *maternal* provisioning or attendance.

Table 3. Change in rearing parents reactive oxygen species (ROS) and lipid peroxidation (LP) levels from courtship to parental care.

	ROS change				LP change			
	Father		Mother		Father		Mother	
Variables	***F***	***P***	***P***	***F***	***F***	***P***	***F***	***P***
Within-subjects effects								
Stage	**18.19**	**<0.001**	**11.54**	**0.002**	0.20	0.66	0.001	0.98
Stage x swapping treatment	2.03	0.17	0.04	0.85	0.01	0.93	0.10	0.76
Stage x chick mass at 15 days	0.17	0.69	0.24	0.63	0.03	0.88	0.67	0.42
Stage x provisioning	0.11	0.74	0.001	0.96	0.62	0.44	0.19	0.67
Stage x attendance	0.02	0.88	0.13	0.73	2.97	0.10	0.60	0.45
Stage x provisioning x swapping	0.03	0.86	0.10	0.76	0.09	0.76	1.23	0.29
Stage x Attendance x swapping	0.30	0.59	0.51	0.49	0.50	0.49	1.90	0.19
Error df final model	28		20		26		22	

Linear models of repeated measures showing the effect of parental investment of rearing parents on the change in levels of reactive oxygen species (ROS) and lipid peroxidation (LP) from courtship to 15 days post hatching (parental care stage). Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion and in bold, variables in the final model.

Table 4. Relationship of rearing and genetic parents reactive oxygen species (ROS) and lipid peroxidation (LP) with offspring ROS and LP.

	Rearing parents				Genetic parents			
	Chick ROS		Chick LP		Chick ROS		Chick LP	
Variables	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Swapping treatment	0.84	0.36	**8.25**	**0.01**	0.84	0.36	**7.09**	**0.01**
Egg volume	0.002	0.97	**5.50**	**0.03**	0.11	0.74	**5.37**	**0.03**
Rate of increase in body mass	0.001	0.97	0.03	0.87	0.15	0.70	0.13	0.72
Weighed begging rate	1.60	0.22	0.89	0.36	0.008	0.93	0.66	0.43
Father ROS/LP$^{(*)}$	0.05	0.82	**11.94**	**0.003**	0.22	0.64	**9.49**	**0.005**
Mother ROS/LP$^{(*)}$	0.04	0.85	**6.10**	**0.02**	0.35	0.56	0.48	0.50
Father ROS/LP$^{(*)}$ x swapping	0.50	0.49	0.77	0.40	0.43	0.52	0.39	0.54
Mother ROS/LP$^{(*)}$ x swapping	0.10	0.77	0.45	0.52	0.01	0.92	1.15	0.30
Error df final model	34		17		33		23	

Linear models showing the relationship of rearing and genetic parents reactive oxygen species (ROS) and lipid peroxidation (LP) with offspring lipid peroxidation and reactive oxygen species, when the chick was 15 days old. Chick weighed begging was calculated as the sum of chick begging rate to mother and father plus one divided by sum of provisioning of father and mother plus one, one was added to avoid zeros in the numerator or denominator. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion and in bold, variables in the final model

(*) Models evaluating the relationship between rearing or genetic parents *ROS* with offspring *ROS*, included only rearing or genetic mother and father *ROS*, measurements of rearing or genetic parents LP were not included in these models. The equivalent procedure was followed when fitting the models evaluating the relationship between rearing or genetic parents *LP* with offspring *LP*.

CAPÍTULO III

Male skin color signals direct and indirect benefits in a species with biparental care

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Original Article

Male skin color signals direct and indirect benefits in a species with biparental care

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Particularly in species with biparental care and low levels of extrapair paternity, sexual traits that honestly indicate phenotypic and genetic quality are expected. We investigated in the brown booby, *Sula leucogaster*, whether gular color displayed by males during courtship is related to direct or indirect benefits to females. We performed a cross-fostering experiment in order to identify the relative contribution of parental care and genetic effects on offspring condition. We found that rearing father gular color was positively related to parental care (offspring attendance and provisioning) and chick body mass increase, whereas the genetic father gular color was related to chick structural growth. Contrary to expectations, females paired to more colorful males laid smaller eggs and did not increase parental care. Interestingly, chicks from genetic mothers with more colorful gulars and chicks that hatched from larger eggs "begged" at higher rates to mothers than to fathers. Overall, the results suggest that male gular color may provide females with reliable information on mate genetic quality and parenting abilities.

Key words: **cross-fostering, feeding behavior, good genes hypothesis, good parent hypothesis, integument coloration, maternal effects, sexual selection, Sula leucogaster.**

INTRODUCTION

Male's parenting ability and genetic quality cannot be evaluated directly during mate choice. As a consequence, females assess potential mates through sexual traits that honestly indicate phenotypic or genetic quality (Kodric-Brown and Brown 1984), and males invest in sexual traits to gain mating advantages (Grafen 1990). Bright colors have been extensively studied as examples of sexual ornaments resulting from female choice (Andersson 1994; Hill 2006) although female ornaments and male mate choice are apparently more common than previously thought (e.g., Torres and Velando 2005; Weiss et al. 2009; Remeš and Matysioková 2013). Particularly, carotenoid-dependent color on fleshy structures has been proposed as an honest trait due to its capacity for being dynamically updated as a function of current changes in immune response, oxidative balance, and nutritional condition (Andersson 1994; Faivre et al. 2003; Bertrand et al. 2006; Velando et al. 2006; Alonso-Alvarez and Galván 2011). Hence, condition-dependent models for the evolution of animal signals state that females paired with colorful males may gain indirect benefits if selected colors indicate male genetic quality (Fisher 1930; Zahavi 1975; Hamilton and Zuk 1982; Kodric-Brown and Brown 1984) or direct benefits if male color indicates ability to acquire good territories or resources for offspring (Kirkpatrick 1985; Heywood 1989; Hoelzer 1989; Price et al. 1993).

The good parent hypothesis predicts that when offspring's fitness is highly dependent on resources provided by the male (i.e., direct benefits), females should prefer more ornamented males if ornaments indicate higher parental care (Kirkpatrick 1985; Heywood 1989; Hoelzer 1989; Price et al. 1993; Kokko 1998; Alonzo 2012). Accordingly, males with carotenoid-dependent colorful ornaments have been found to feed offspring at higher rates and/or higher quality diets (García-Navas et al. 2012; Pagani-Núñez and Senar 2014, but see Mitchell et al. 2007), and when environmental and genetic factors have been differentiated, more ornamented males produced offspring in better condition, regardless of the color of the genetic father (Senar et al. 2002; Velando et al. 2005; but see Hadfield et al. 2006). Conversely, the good genes hypothesis predicts that, when the male genetic quality has a significant influence on offspring fitness, females assess potential mates through traits that indicate indirect benefits (Hamilton and Zuk 1982; Barber et al. 2001; Eilertsen et al. 2009). However, whether sexual traits may be honest indicators of direct or indirect benefits may also depend on particular features of the mating system or life-history

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traits (Kokko 1998; Kokko et al. 2002). For instance, in species with biparental care and limited opportunities of extrapair copulations, sexual traits are expected to indicate both genetic quality and condition-dependent parental investment simultaneously (Kokko 1998; Velando et al. 2006; Pickett et al. 2013).

Life-history theory predicts that individuals adjust investment into current reproduction in response to the expected fitness returns (Trivers 1972; Stearns 1992). Hence, if sexually selected male traits provide honest information of male genetic quality and/or parenting abilities, females are expected to invest more when paired with an attractive male (Burley 1988; Sheldon 2000; Qvarnström and Price 2001) or to compensate when attractive males provide less paternal care (Mousseau and Fox 1998; Gowaty et al. 2007). Furthermore, females might prepare offspring for the expected conditions during development by modifying prenatal nourishment (Mousseau and Fox 1998) or by modulating through allocation of maternal hormones in the yolk, the offspring solicitation behavior that is used by parents to adjust their level of parental care (Groothuis et al. 2005; Goodship and Buchanan 2006; Hinde et al. 2009; Noguera et al. 2013). Such maternal effects (i.e., nongenetic contributions of the mother to the offspring) may increase offspring growth and survival (Gowaty et al. 2007), potentially confounding estimates of direct or indirect benefits of mating with attractive males (Gil and Graves 2001; Qvarnström and Price 2001). Females paired with more ornamented males have been found to adjust reproductive investment by increasing egg size (Velando et al. 2006; Giraudeau et al. 2011; Alonso-Alvarez et al. 2012), chick provisioning (Johnsen et al. 2005), or modifying the eggs' biochemical composition such as androgens (Gilbert et al. 2006; Dentressangle et al. 2008; Gilbert et al. 2012), antibodies (Groothuis and Schwabl 2008), and carotenoids (McGraw et al. 2005). Hence, females might adjust pre- and posthatching maternal effects to variable family or ecological conditions.

The brown booby (*Sula leucogaster*) is a tropical long-lived seabird with a socially monogamous breeding system and low occurrence of extrapair copulations (Nelson 1978; Gañan et al. 2014). In the brown booby colony of Islas Marietas, 18% of males from 33 observed pairs engaged in extrapair copulations, and no extrapair copulations by females were recorded (Gañan et al. 2014). Males and females incubate during roughly 42 days (±3 days) a modal clutch size of 2 eggs and feed the offspring by transferring fish directly into their mouths during an approximate 3-month period before a chick reaches independence (Nelson 1978). Brown boobies show obligate siblicide; consequently, even when both chicks hatch, the older one typically eliminates the younger one during the first few days of life (Drummond et al. 2003; Osorno and Drummond 2003). In addition to the costly investment on food provisioning, male parental effort probably plays a key role during incubation and chick rearing because neither the eggs nor the young chicks during 3–4 weeks posthatching can be left unattended (Nelson 1978; Tershy and Croll 2000). Therefore, females might benefit from choosing mates that honestly indicate their quality and probable parental effort, and when paired with more attractive males, females might gain by increasing their own investment on offspring or by preparing offspring to match future rearing conditions.

Brown booby males and females from the Eastern Pacific colonies differ in the color of their plumage and their bare skin on the face (i.e., cheek and gular skin) and feet. Male plumage is white on the forehead and fore crown, shading into pale brownish gray on the neck, whereas females show brown plumage on the head and neck (Nelson 1978). During courtship, males display green-bluish coloration on the face and feet, whereas females display yellow-greenish coloration on the face and feet. Recently, we found that gular and feet color during courtship is carotenoid dependent (Montoya B, Torres R, unpublished data), suggesting that color might be an honest indicator of condition.

To evaluate whether brown booby females obtain direct and/or indirect benefits by mating with a male with greener gular coloration, we performed a cross-fostering experiment designed to separate genetic from environmental parental effects on offspring growth rate (body mass and ulna length increase between hatching and age 15 days). If the male's gular color is an indicator of paternal care, we expected that his green gular chroma positively covaried with time spent in the nest (i.e., offspring attendance), frequency of chick provisioning, and rate of ulna and mass increase of the chicks he is raising. If male gular color indicates his genetic quality, we expected that it would positively covary with the growth rate of ulna length and mass-gain rate of his genetic offspring. Furthermore, if females adjust their reproductive effort to the partner's gular color, we expected that the greener her partner's gular skin, the greater the probability that she establishes a clutch, the earlier she completes it, the larger her eggs, and the greater her offspring attendance and provisioning. Finally, if females prepare their offspring for expected conditions during the rearing period (Mousseau and Fox 1998), and sexual signals are reliable indicators of the provisioning capacity of parents, we expected that chick begging rate would covary positively with the green chroma of their genetic father.

METHODS

The study was conducted from June to October 2011 in the brown booby breeding colony at Isla Larga, Parque Nacional Islas Marietas, Nayarit, Mexico (20°41′N 105°36′W) in the Eastern Pacific.

During courtship, 95 brown booby pairs were captured by night lighting (Velando et al. 2006), and both members of the pair were individually marked with a white polymethylmethacrylate numbered leg band (Interrex, Poland). For captured birds, we measured body mass (±20 g), ulna length (±1 mm), and the color of the gular using a portable spectrophotometer that determines the reflectance at 10-nm intervals from 360 to 740 nm (MINOLTA CM 2600d, Osaka, Japan). The blue-green gular color displayed by males has 2 main peaks, one at 360 (within the UV range) and the other at 540 nm (within the green range). In contrast, female gular color peaks at 520 and 620 nm (within the yellow range). Hence, our color measurements included the most important features of the gular reflectance spectra of males and females. The gular color of each individual was automatically obtained as the mean of 3 sequential measurements roughly 2 cm apart placing the spectrophotometer at 90° from the gular skin surface. Measurements were collected using a target mask (diameter 8 mm) that avoids the entrance of external light. The spectrophotometer uses diffuse illumination from 3 internal pulsed xenon lamps set at 10°. The apparatus was calibrated daily against a white target, and data were downloaded using the OnColor software (CyberChrome, Inc.). All measurements for a pair were completed in roughly 10 min, and the birds were returned to their nest site, which was marked with a numbered flag. Nests were checked daily between 18:00 and 20:00 h to determine laying date. On the day a new egg was laid, we measured its mass (±0.1 g) and volume in mm^3 (length × width^2 × 0.51/1000, Hoyt 1979). Fifty-three from the 95 pairs established a clutch, yet one clutch did not fulfill the requirements to be swapped and was, therefore, excluded from the cross-fostering experiment.

Cross-fostering

We performed a cross-fostering experiment in order to separate the genetic from the environmental effects on the offspring condition. This experimental design does not allow us to distinguish between genetic and premanipulation maternal effects. On average, 15 days (±3 days) after the first egg was laid, complete clutches were randomly assigned to either the experimental (n = 28 two-egg and 3 one-egg clutches) or the control group (n = 15 two-egg and 6 one-egg clutches). In the experimental group, clutches of similar laying date (±3 days, range: 0–5 days), clutch size (1 or 2 eggs), and within our sample, the greatest differences in males' gular color at 540 nm (the peak wavelength within the range of green reflectance in our sample) were swapped (male gular color between males of swapped clutches, $r_p = -0.50$, $P = 0.008$). In the control group, the swapping procedure was simulated, and the clutch was returned to its original nest after 5 min, a similar amount of time that swapping clutches took in the experimental group. To avoid nest desertion, 2 hen eggs were left on the nest while the clutch was transported either between nests (experimental) or within the same nest (control). After swapping, nests were visited every 5 days during incubation and daily after the first chick hatched to register survival until chicks were 15 days old. At the age of 1, 5, 10, and 15 days, chicks' body mass (±1 g) and ulna length (±1 mm) were measured. At the age of 15 days, 1 mL blood sample was taken from the brachial vein and stored at −70 °C in order to determine the sex of the chick using the procedure of Griffiths et al. (1998).

From the 52 swapped clutches: in 40 cases, only 1 egg hatched (37 were first or single eggs and 3 were second eggs), in 7 cases, both eggs hatched, yet all second-hatched chicks died during the first 2 days after hatching, and in 5 cases, no hatching occurred. Finally, in 8 cases, the surviving chick died before day 15. Hence, the sample for analyses of parental effort was 39 nests with a single chick that survived up to age 15 days.

Behavioral sampling

Four observers sat without a blind at a distance of roughly 3–6 m (brown boobies during the chick rearing period are extraordinarily tolerant of human presence; Drummond et al. 2003) from focal nests and recorded 1) the absolute occurrence of feeding by the male or the female (when a parent places the bill above the chick's head and the chick places its head into the parent's bill), 2) occurrence of chick begging to the male or the female (when the chick raises the head and vocalizes with a "tac-tac" sound) in every 5-min intervals (1-0 records), and 3) the minute and second of arrivals to and departures from the nest by each parent to estimate offspring attendance (for detailed descriptions of behaviors, see Nelson 1978). After a period of training, interobserver reliability was ≥90% at the beginning of the study.

Behavioral observations were carried out at days 1, 5, 10, and 15 (±3 days) after the first chick hatched, from 07:00 to 09:00 h and from 17:00 to 21:00 h, the 2 periods of greater diurnal parental activity at Isla Larga (Montoya B, unpublished data). Observers were unaware about which experimental treatment focal nests belonged. Observations when the chick was 1, 5, 10, and 15 days of age were pooled together, and offspring attendance (total time with the chick) by the rearing father or mother, the absolute number of feedings by the rearing father or mother (food provisioning), and begging rate to the rearing father or the mother (sum of total begging events to the father or the mother/sum of total time the parent was present at the nest) were calculated for the analyses.

Statistical analysis

From the spectral curves, we calculated the males' UV chroma (sum of reflectance 360–400 nm/sum of total reflectance 360–740 nm), blue chroma (sum of reflectance 430–470 nm/sum of total reflectance), and green chroma (sum of reflectance 480–550 nm/sum of total reflectance) and the females' maximum reflectance value within the range of 360–740 nm (hereafter female gular color). The gular color during courtship of 10 females was missed in the field. As female's gular color did not differ between courtship and 15 days after hatching (paired t-test = −0.87, P = 0.38, n = 29; Montoya B, Torres R, unpublished data), gular color of 4 females captured during the chick rearing period was used as estimates of their color during courtship. In order to summarize male color variables, for the analyses post cross-fostering, we performed a principal component (PC) analysis. The analysis resulted in 3 PCs that explained 59.86%, 34.38%, and 5.74%, respectively (eigenvalues: PC1 = 1.79, PC2 = 1.03, and PC3 = 0.17). For the analyses post cross-fostering, we used the PC1 (hereafter male gular color) that varied from negative values, indicating higher green chroma, to positive values indicating higher UV and blue chromas (factor loadings for PC1—green chroma: −0.60, UV chroma: 0.71, and blue chroma: 0.34). For clarity, only for the figures, PC1 was multiplied by −1; hence, negative values indicate higher UV chroma and positive values indicate higher green chroma.

Laying probability and hatching success were analyzed using generalized linear models with binomial error distribution and a logit link function. Total clutch volume (sum of all eggs' volume in the clutch) and first egg volume were analyzed with general linear models with normal error distribution. First egg volume was analyzed because in the brown booby, first eggs probably have a greater reproductive value than second eggs: in a previous study, 7% of chicks that fledged came from second eggs (Tershy et al. 2000). Similarly, in our study, only 7% (3/43) of the chicks that survived until day 15 posthatching came from second eggs. Initial models of laying probability, clutch volume, and first egg volume included the gular color (UV, blue and green chromas) of the genetic father, the gular color of the genetic mother, and male and female body mass. Initial model of hatching success included the swapping treatment (i.e., experimental or control), the gular color (PC1) of the genetic and rearing father, and the gular color of the genetic and rearing mother.

The analyses of offspring attendance were performed using general linear models with normal error distribution. Food provisioning was analyzed with generalized linear models with a log link function and Poisson error distribution for male provisioning and, to control for overdispersion, quasi-Poisson error distribution for female provisioning; additionally, in both analyses, an F-test, a more strict test, rather than a chi-square test was used to compensate for overdispersion (Crawley 2002). Initial models included gular color of the genetic and rearing father and mother, swapping treatment, sex of the chick, mass of the chick at hatching, laying date, and the interaction between the gular color of the genetic and the rearing father. Additionally, chick's begging rate was included in the analyses of provisioning rate. To investigate the effect of male phenotype on offspring increase in body mass and ulna length, we used repeated-measures models that included the chick identity as the subject term and the age (body mass or ulna length at hatching and age 15 days) as the repeated measure. The models included age as a within-group fixed factor and swapping treatment and sex of the chick as fixed factors. The gular color of the genetic and rearing father, the genetic and rearing mother, laying date, and egg volume were included as covariates. To evaluate the effect of our estimates of parental care on offspring growth, feeding rate (number of feedings/total time the parent was present at the nest) by the rearing father and mother was also included in the models. In the model of ulna length increase, one influential point was excluded from the analysis (id15, outlier test = −2.35, $P = 0.02$). For the analysis of chick body mass, the change in ulna length between hatching and age 15 days was included as covariate.

To investigate if chick's begging rate was related to the male's gular color, we fitted a repeated-measures model with chick identity as the subject term and begging rate to the father and to the mother as the repeated measure. The model included the sex of the rearing parent as the within-group fixed factor and the swapping treatment and the sex of the chick as fixed factors. The parents feeding rate, the gular color of the genetic and rearing father and mother, the volume of the egg, and the mass at hatching were included as covariables. For all analyses, final models were obtained by backward deletion procedure of nonsignificant terms. All analyses were carried out in R 3.1.0 (Development Core Team 2014) or SAS software 9.0 (SAS Institute 1999).

RESULTS

Do females obtain direct or indirect benefits by mating with a more colorful male?

Cross-fostering experiment

Hatching success was unrelated to the color of the genetic or rearing father or mother (genetic father: $\chi^2 = 0.04$, $P = 0.82$; rearing father: $\chi^2 = 1.17$, $P = 0.27$; genetic mother: $\chi^2 = 0.41$, $P = 0.51$; rearing mother: $\chi^2 = 0.22$, $P = 0.63$). No differences in hatching success between swapping treatments were found ($\chi^2 = 0.15$, $P = 0.69$, $n = 49$ pairs).

According to our prediction of direct benefits, rearing fathers with greener gulars invested more in offspring attendance and food provisioning than less colorful fathers. Rearing fathers with greener gulars spent 30% more time attending the chicks than less colorful fathers (11.47 ± 0.81 vs. 8.84 ± 0.97 h; Table 1 and Figure 1a). Offspring attendance by the rearing father was unrelated to the gular color of the genetic father (main effect and interaction of gular color of rearing × genetic father, Table 1). However, rearing fathers spent more time with the chick when their mate's gular color was duller yellow ($\beta = -0.38 \pm 0.13$), the genetic mother gular color was brighter yellow ($\beta = 0.34 \pm 0.14$), and when the chick was lighter at hatching ($\beta = -0.26 \pm 0.10$; Table 1). Other variables in the model did not have a significant effect on offspring attendance (Table 1). On average, males spent less time with chicks than females (males, 9.98 ± 0.67 h; range: 1.67–16.67 h; females, 12.74 ± 0.57 h; range: 6.0–19.67 h; paired *t*-test = −2.57, $P = 0.01$, $n = 39$), and within pairs, offspring attendance by fathers and mothers was negatively correlated ($r_p = -0.48$, $P = 0.002$).

Fathers with greener gulars fed chicks 35% more often than less colorful males (7 ± 1.28 vs. 4.54 ± 0.92 feedings/observation period; Table 1 and Figure 1b). In addition to the effect of its own color, rearing fathers provided more food to offspring whose genetic father had a greener gular ($\beta = 0.12 \pm 0.05$); the interaction between genetic and rearing father gular color was not significant (Table 1). Also, provisioning by rearing fathers was positively related to the chicks' begging rate ($\beta = 0.07 \pm 0.01$, Table 1). Other variables in the initial model were unrelated to provisioning by the rearing father (Table 1). Provisioning by males and females did not differ (males 5.62 ± 0.39 vs. females 5.12 ± 0.34 feedings/observation period; paired *t*-test = −0.37, $P = 0.71$, $n = 39$), and male and female provisioning was not related ($r_p = -0.04$, $P = 0.76$).

At the age of 15 days, chicks from rearing fathers with greener gulars gained on average 9% more body mass than chicks from

Table 1

Male gular color during courtship and offspring attendance and food provisioning

	Rearing father			
	Attendance		Provisioning	
Variable	*F*	*P*	*F*	*P*
Swapping treatment	2.10	0.15	1.73	0.18
Chick sex	2.01	0.16	0.0007	0.89
Laying date	3.99	0.055	2.70	0.10
Body mass at hatching	**6.01**	**0.02**	2.43	0.93
Genetic father color	0.01	0.90	**7.89**	**0.004**
Rearing father color	**5.29**	**0.02**	**12.79**	**<0.001**
Genetic mother color	**5.87**	**0.02**	2.56	0.10
Rearing mother color	**8.26**	**0.007**	0.76	0.38
Color genetic × rearing father	0.76	0.39	1.95	0.16
Begging rate to father	—	—	**18.46**	**<0.001**
Error df of final models	32		34	

Offspring attendance was analyzed with general linear models with normal error distribution, whereas food provisioning was analyzed with generalized linear models with Poisson error distribution. The table shows *F* and *P* values from variables in the initial model at the moment of their exclusion, and in bold, *F* and *P* values from variables in the final model. df, degrees of freedom.

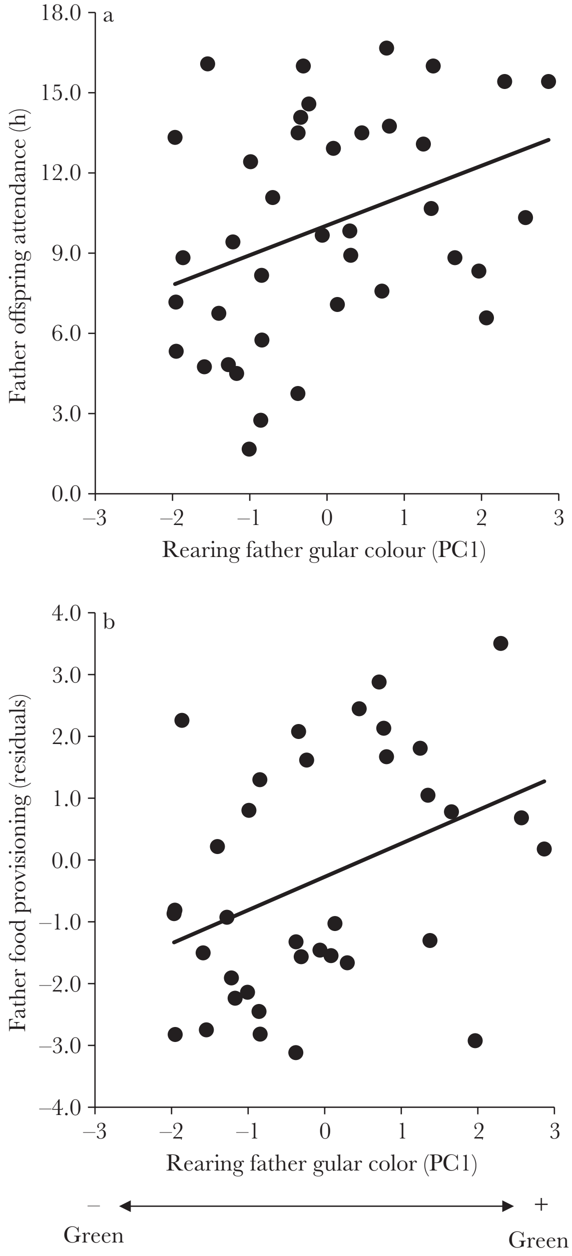

Figure 1
Rearing fathers with lighter green gulars during courtship spend more time attending the offspring and provide more food. (a) Rearing father gular color (PC1) and offspring attendance during the first 15 days posthatching. (b) Rearing father gular color (PC1) and chick provisioning. Male color is expressed as the first principal component (PC1) from a PC analysis: positive values indicate higher green chroma and negative values indicate higher UV chroma.

rearing fathers with less colorful gulars (Table 2 and Figure 2a). The genetic father's gular color and its interaction with the rearing father gular color were not significant (Table 2). Body mass increase was positively related to the increase in ulna length between hatching and 15 days ($\beta = 1.82 \pm 0.79$); yet, chicks from larger eggs gained less body mass ($\beta = -2.70 \pm 1.55$, Table 2). The rate of feeding by the father ($\beta = 5.04 \pm 2.71$) and the mother ($\beta = 21.45 \pm 6.11$) had a positive effect on chick body mass gain (Table 2). Other variables in the initial model did not have a significant effect on the chick's body mass increase (Table 2).

Interestingly, ulna growth from hatching to 15 days was related to the genetic father gular color, but not to the rearing father gular color (main term and interaction genetic father gular color × rearing father gular color, Table 2). Genetic fathers with greener gulars produced chicks 9% larger than chicks from fathers with duller gular color (Table 2 and Figure 2b). Also, there was a negative relationship between the chick structural growth and the rearing mother gular color ($\beta = -0.30 \pm 0.19$; Table 2). Surprisingly, feeding rate by the father and the mother did not have a significant effect on the chick ulna growth (Table 2). Other variables in the initial model did not have a significant effect on the chick's ulna length increase (Table 2).

Do females adjust breeding effort according to male gular color?

The probability to establish a clutch was unrelated to the male gular color, female gular color, and male and female body mass (male green chroma, $\chi^2 = 3.24$, $P = 0.072$; all other terms in the model, $P > 0.16$, $n = 83$ pairs). Similarly, total clutch volume was unrelated to the gular color and body mass of males and females during courtship and to laying date (all $P > 0.17$). However, females paired with males with greener gulars laid smaller first eggs (male green chroma: $F_{1,45} = 4.19$, $P = 0.046$; Figure 3), and egg volume increased with laying date ($F_{1,45} = 10.84$, $P = 0.002$; all other terms in the model, $P > 0.11$).

Cross-fostering experiment

Contrary to our expectation, females did not increase offspring attendance or food provisioning when mated to a more colorful male. Offspring attendance and food provisioning by rearing mothers were unrelated to the color displayed during courtship by the genetic or the rearing father (main effects and interaction of color of genetic × rearing father, Table 3). Rearing mothers with brighter yellow gulars spent more time with the chick ($\beta = 0.33 \pm 0.12$), yet attending decreased when the genetic mother had a brighter yellow gular ($\beta = -0.28 \pm 0.12$); other variables in the model of offspring attendance by the rearing mother were not significant (Table 3). Provisioning by rearing mothers was unrelated to the gular color of the genetic and rearing father or the genetic and rearing mother (Table 3). Provisioning by mothers increased when the intensity of begging increased ($\beta = 0.15 \pm 0.05$) and when chicks were heavier at hatching ($\beta = 0.11 \pm 0.03$). Additionally, females in the experimental treatment increased food provisioning compared with control females (swapping treatment, Table 3). All other variables in the model were not significant (Table 3).

Is offspring begging rate related to the father's gular color?

Chick's begging rate to the father or the mother was unrelated to the gular color displayed during courtship by the genetic (color of genetic father × parental sex: $F_{1,26} = 0.32$, $P = 0.57$) or rearing father (color of rearing father × parental sex: $F_{1,26} = 0.42$, $P = 0.52$). However, chicks begged at higher rates to mothers than to fathers when the genetic mothers had brighter yellow gular and when they came from larger eggs (Table 4 and Figure 4). Additionally, overall begging rate was positively related to parental feeding rate ($\beta = 1.11 \pm 0.22$) and negatively related to the gular color of the rearing mother ($\beta = -0.21 \pm 0.10$) and the chick mass at hatching ($\beta = -0.26 \pm 0.07$;

Table 2

Male gular color during courtship is related to direct and indirect benefits on chick growth

Variable	Chick growth Body mass *F*	*P*	Ulna size *F*	*P*
Within-subjects effects				
Age × swapping treatment	1.19	0.28	0.001	0.98
Age × chick sex	0.76	0.39	0.05	0.82
Age × ulna change (1st day − 15th day)	**26.25**	**<0.001**	—	—
Age × egg volume	**6.80**	**0.01**	0.47	0.84
Age × laying date	2.91	0.09	0.04	0.49
Age × genetic father color	1.95	0.17	**6.82**	**0.01**
Age × rearing father color	**5.99**	**0.02**	0.02	0.88
Age × genetic mother color	0.14	0.71	1.84	0.18
Age × rearing mother color	0.05	0.81	**7.40**	**0.01**
Age × father feeding rate	**4.86**	**0.03**	2.66	0.11
Age × mother feeding rate	**5.09**	**0.03**	2.53	0.12
Age × color genetic × rearing father	0.36	0.55	0.17	0.68
Error df of final models	29		32	

Chick growth was analyzed with repeated-measures models that included the chick identity as the subject term and the age (hatching and age 15 days) as the repeated measure. The table shows *F* and *P* values from variables in the initial model at the moment of their exclusion, and in bold, *F* and *P* values from variables in the final model. df, degrees of freedom.

Table 4). The swapping treatment ($F_{1,25} = 2.34$, $P = 0.13$) and sex of the chick ($F_{1,25} = 0.48$, $P = 0.49$) were not significant.

DISCUSSION

Do females obtain direct or indirect benefits by mating with a more colorful male?

In species with biparental care and low levels of extrapair copulations, it is expected that sexual traits may signal both direct and indirect benefits (Kokko 1998). Accordingly, we found that brown booby females may obtain direct and indirect benefits by paring with males with greener gulars. Through a cross-fostering experiment, we showed that gular color displayed during courtship by rearing fathers was positively related to male parental care and chick body mass increase, whereas genetic father gular color was positively related to chick structural growth.

Rearing fathers with greener gulars spent 30% more time next to the chick and fed chicks 35% more often than less colorful fathers. In the brown booby, gular and feet color displayed during courtship is carotenoid dependent (Montoya B, Torres R, unpublished data). Carotenoid-based coloration has become a focus of many studies of sexual selection owing to its important role in condition-dependent signaling. Because vertebrates acquire carotenoids through the diet, it has been suggested that carotenoid coloration has strong diet dependence and may function as an indicator of nutritional state (Hill and Montgomerie 1994; Hill et al. 2002; McGraw et al. 2003) and foraging efficiency (Senar and Escobar 2002; Karino et al. 2005; Casagrande et al. 2006; García-Navas et al. 2012). Thus, if colorful brown booby males are more efficient foragers and have better nutritional condition, they probably can spend more time in parental activities and provide more and/or higher quality food to their offspring. Overall, the results suggest that males that display greener gulars during courtship indicate higher paternal care.

In addition to the benefits via the potential increase in offspring fitness as a result of higher paternal abilities, fathers that invest more in offspring attendance and food provisioning may reduce female load of maternal duties, which may positively impact female condition (Christe et al. 2012). In our study, the frequency of food provisioning by males and females partners was unrelated. However, female offspring attendance decreased when their mates spent more time with the chick, suggesting that females may benefit by the extra offspring attendance performed by more colorful males.

Offspring of genetic fathers with greener gulars had greater increase in size, and after controlling for structural size, offspring of rearing fathers with greener gulars showed a greater increase in body mass than offspring from duller fathers. Feeding effort by the rearing mother and father had a strong positive effect on the magnitude of chick body mass gain, indicating a direct effect of parental provisioning on the offspring condition. However, structural grown was unrelated to feeding effort by rearing parents, supporting the idea of genetic effects on the rate of structural growth. Size and body mass at early stages have been found to increase fledging survival and probability of recruitment (Naef-Daenzer et al. 2001). Furthermore, body mass in early stages of development has shown to be related to the adult phenotype resulting in offspring with more colorful carotenoid-dependent sexual ornaments (Olsson et al. 2002; Walker et al. 2013). Thus, by mating with males with greener gulars, brown booby females may obtain direct and indirect benefits by increasing offspring size and condition.

Do females adjust breeding effort according to male color?

Our prediction that females would increase breeding effort when their partners displayed greener gular skin was not supported by the results. The probability to establish a clutch, female offspring attendance, and food provisioning were unrelated to male gular color, and contrary to our expectation, females paired with males with greener gulars laid smaller first eggs. Additionally, egg volume was positively related to laying date. Egg volume

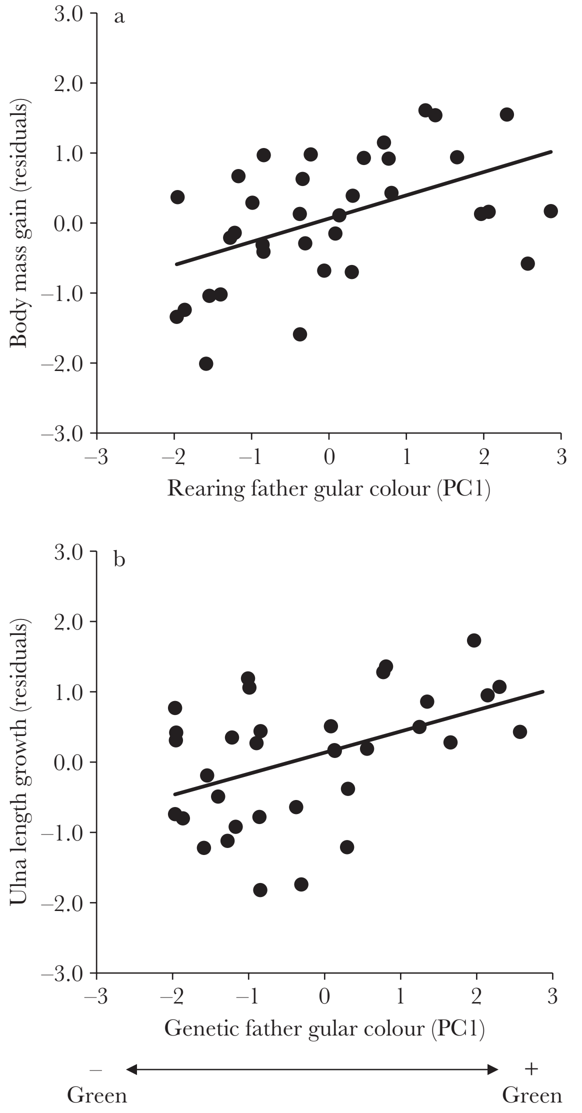

Figure 2
Male gular color during courtship is related to direct and indirect benefits on chick growth. (a) Chick body mass gain from hatching to age 15 days and rearing father gular color during courtship. The residuals of the linear model controlling for ulna length increase are shown. (b) Chick ulna growth from hatching to age 15 days and genetic father gular color during courtship. The residuals of the linear model controlling for the rearing mother gular color are shown. Male color is expressed as the first principal component (PC1) from a PC analysis: positive values indicate higher green chroma and negative values indicate higher UV chroma.

and laying date have been extensively studied because of their influence in breeding success. For instance, egg volume has been positively related to hatching success and body mass of hatchlings (D'Alba and Torres 2007, reviewed in Krist 2011), while laying later in the breeding season is typically related to a decline in the phenotypic quality of breeding pairs and/or overall breeding conditions (reviewed in Verhulst and Nilsson 2008). In the brown booby, the fact that females lay larger eggs when paired to a less ornamented male and later in the season suggest that females might be compensating for a bad genetic and environmental quality mate (Bolund et al. 2009).

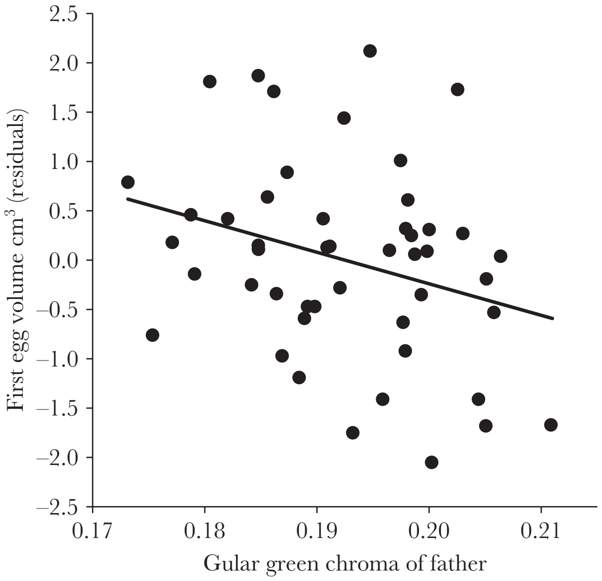

Figure 3
Females paired with males with lighter green gulars lay smaller first eggs. The residuals of the linear model controlling for laying date are shown.

Is offspring begging rate related to the father's gular color?

We did not find any evidence supporting the prediction that offspring from genetic fathers with greener gulars would beg at higher rates. Offspring begging rate was unrelated to the color of the genetic father. However, chicks beg at higher rates to mothers than to fathers when the genetic mother had a brighter yellow gular and when the chick came from a larger egg. It has been suggested that when parents differ in their reproductive tasks, with one parent providing more food than the other, offspring are expected to differ in the intensity of begging to each parent (Roulin and Bersier 2007). In our study, mean food provisioning by males and females did not differ, and we have no evidence that brown booby parents differ substantially in their reproductive tasks. Because females laid larger eggs when mated with less colorful males and later in the season, presumably under poorer breeding conditions, it is possible that chicks from larger eggs beg at higher rates because they were hungrier. The reason why chicks beg at higher rates to mothers than to fathers remains unclear. However, if females with brighter yellow gulars are more able to compensate than males when breeding conditions deteriorate (e.g., Velando and Alonso-Alvarez 2003), it is possible that chicks increase begging when the mother is at the nest. In our study, begging behavior was highly correlated to provisioning by both parents; nevertheless, the relationship between begging and provisioning was more pronounced for females than for males, suggesting that females might be more responsive to offspring solicitation. Future studies are needed to investigate the potential mechanisms underlying variation of begging behavior with egg volume and female gular color as well as the fitness consequences for the chick and the parents.

In conclusion, our results support the idea that in species with biparental care and low levels of extrapair copulations, sexual traits may signal direct and indirect benefits. We found that fathers with greener gulars spent more time attending the offspring and provided more food. Furthermore, the chick structural growth was related to the genetic father gular color, whereas

Table 3

Female gular color during courtship and offspring attendance and food provisioning

	Rearing mother			
	Attendance		Provisioning	
Variable	*F*	*P*	*F*	*P*
Swapping treatment	0.21	0.64	**5.44**	**0.02**
Chick sex	0.85	0.36	2.82	0.10
Laying date	0.013	0.91	0.81	0.37
Body mass at hatching	0.03	0.85	**10.38**	**0.002**
Genetic father color	0.001	0.97	2.53	0.12
Rearing father color	2.16	0.15	0.001	0.96
Genetic mother color	**4.82**	**0.03**	0.006	0.93
Rearing mother color	**7.47**	**0.01**	0.02	0.88
Color genetic × rearing father	0.03	0.84	0.22	0.63
Begging rate to mother	—	—	**9.54**	**0.004**
Error df of final models	34		33	

Offspring attendance was analyzed with general linear models with normal error distribution, whereas food provisioning was analyzed with generalized linear models with quasi-Poisson error distribution to account for overdispersion. The table shows *F* and *P* values from variables in the initial model at the moment of their exclusion, and in bold, *F* and *P* values from variables in the final model. df, degrees of freedom.

Table 4

Chick begging rate to the rearing father and mother (*n* = 34 chicks)

	Begging rate		
Variable	df	*F*	*P*
Between-subjects effects			
Genetic mother color	1,29	6.84	0.01
Rearing mother color	1,29	4.38	0.045
Egg volume	1,29	6.03	0.02
Body mass at hatching	1,29	11.82	0.001
Parental sex	1,28	7.73	0.009
Parents feeding rate	1,28	25.25	<0.0001
Within-subjects effects			
Parental sex × genetic mother color	1,28	5.62	0.02
Parental sex × egg volume	1,28	4.83	0.03

Begging rate was analyzed with repeated-measures models that included the chick identity as the subject term and the chick's begging rate to the father and to the mother as the repeated measure. The table shows the final model after stepwise backward procedure for model simplification, and nonsignificant terms are reported in the text. df, degrees of freedom.

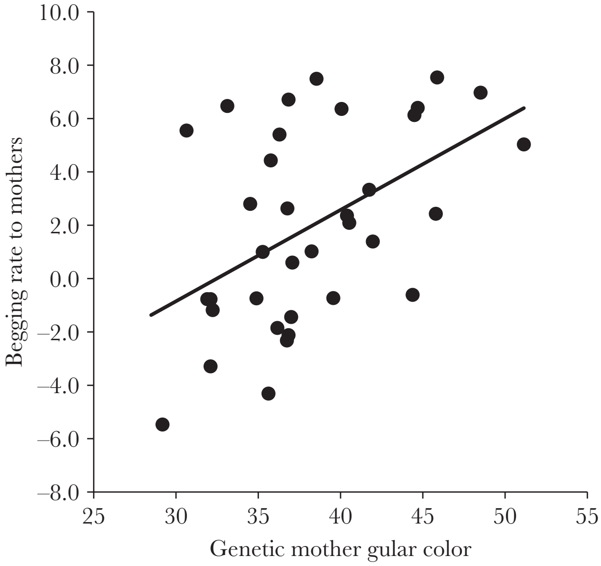

Figure 4

Chick begging rate difference to the mother versus father in relation to the genetic mother gular color. Only for illustration purposes, the difference between the begging rate to the rearing mother and the rearing father is shown (begging rate to mother − begging rate to father).

body mass increase was related to the rearing father gular coloration. Interestingly, when mated with less colorful males or when laying late in the season, females laid larger eggs, suggesting a female compensatory strategy when the partner's genetic or environmental quality is low. Overall, the results suggest that in the brown booby, male gular color may provide females with reliable information on mate genetic quality and parenting abilities.

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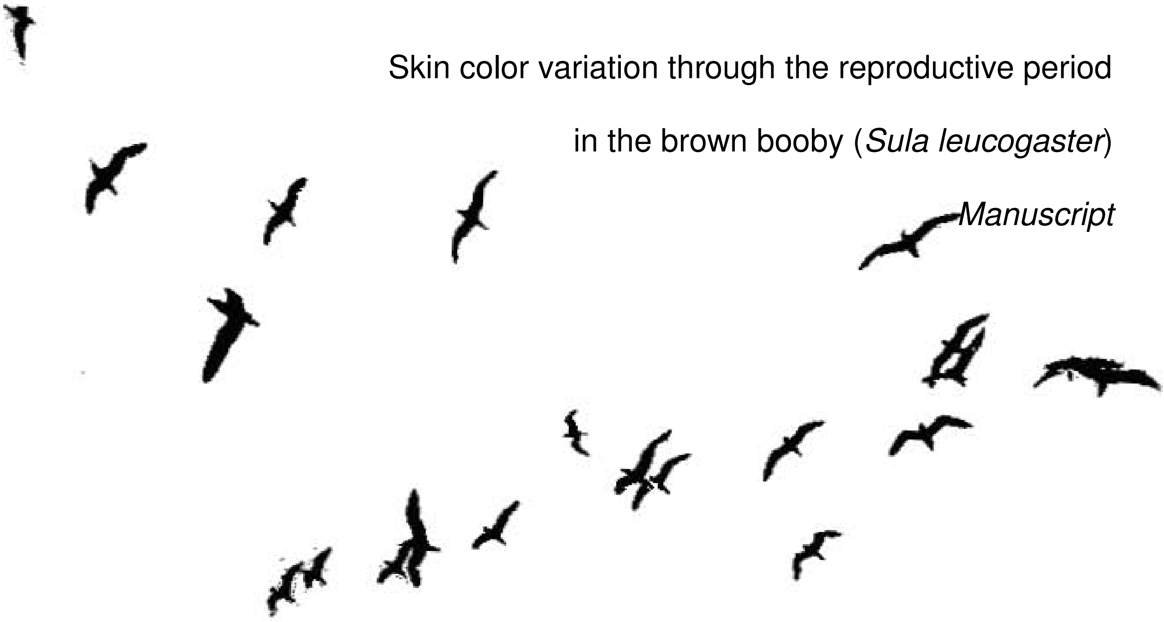
CAPÍTULO IV

Skin color variation through the reproductive period in the brown booby (*Sula leucogaster*)

Manuscript



Skin color variation through the reproductive period in the brown booby (*Sula leucogaster*)

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Running title: Skin color variation

ABSTRACT

Individual differences in the capacity to invest in a reproductive attempt cannot be evaluated directly by potential partners, favoring thereby the evolution of signaling mechanisms. Additionally, individual’s capacity to invest can vary through the reproductive period and signals might provide information about such variation. We studied color variation in gular skin of male and female brown boobies (*Sula leucogaster*). Male gular UV, blue, green and red, and female gular blue and green varied through the reproductive stages (courtship, hatching or parental care). Capture date was associated with a decline in male and female gular green, irrespective of the specific reproductive stage, suggesting environmental variation associated with color change. Gular total brightness was repeatable over the reproductive season in both sexes. However, chromatic components of gular color in males were not repeatable between the different reproductive stages, and in females only gular UV and green exhibited significant repeatability. When gular color was estimated using a model that considers specific avian visual system characteristics (avian visual model) no significant differences of female gular photon reception with reproductive stage or capture date were identified, whereas male gular photon reception did vary with reproductive stage and marginally with capture date. Male and female gular photon catch was higher in individuals which established a clutch. Finally, male, but not female gular photon reception showed some repeatability over the reproductive season. Overall, results suggest that the mechanisms of male and female gular color production might be similar, and gular color may have a role as informative signal in both sexes.

Key words: Dynamic signals, carotenoid-dependent, condition-dependent

Individuals invest in reproduction based on the magnitude of benefits expected, and such benefits have been suggested to be, at least partially, associated with the quality of the reproductive mate (Stearns 1992; Andersson 1994). However, during courtship, individual quality cannot be assessed directly by potential partners, favoring thereby the evolution of signaling mechanisms (Zahavi 1975; Kodric-Brown & Brown 1984; Grafen 1990). Signals of quality can remain static over time, or vary dynamically in the course of days or even hours, yet conserving their informative value in changing environments (Bro-Jørgensen 2009). The evolution of dynamic traits that mirror the impact of environmental changes on individual condition is expected when signal production depends more on environmental than on genetic variation, or when environmental change has different effects on the success of different genotypes (genotype by environment interaction; Higginson & Reader 2009). Therefore, the evolution of dynamic signals is expected especially in species where reproductive investment and success are heavily affected by environmental variation.

Condition-dependent models for the evolution of animal signals propose that in order to evolve into honest indicators of benefits provided to potential mates, signal production must be associated with individual condition or entail differential costs to signalers depending on their quality (Zahavi 1975; Grafen 1990). Variation in the cost an individual pays to express the signal, or in the individual's capacity to deal with such cost, might affect signal expression resulting in a dynamic change of it. How dynamically a signal varies can be determined by the degree at which the change can mirror variations in the costs associated to its expression without compromising the reliability (Bradbury & Vehrencamp 1998). Unlike some structures used as sexual signals such as horns or colored feathers, fleshy bare structures have been found to change over short periods of time (Rosen & Tarvin 2006; Velando *et al*. 2006), minutes when dependent on blood irrigation (Negro *et al*. 2006), or days when pigment dependent, as pigments can be mobilized in a facultative way (Lozano 1994). Consequently, due to their potential to vary dynamically, skin colors might provide updated information about current individual condition (Faivre *et al*. 2003).

Carotenoid-dependent traits have received great attention during the last two decades, because these molecules cannot be synthesized *de novo* by vertebrates, being principally available for good foragers (Hill 1994; Olson & Owens 1998; Casagrande *et al*. 2006). Additionally, once acquired through the diet, the use of carotenoids in ornaments relies on at least three processes: the efficiency to absorb and mobilize them, the necessity to allocate them to other vital functions such as the immune or antioxidant responses, and the capacity to deal with their toxic potential (Lozano 1994; Olson & Owens 1998; Møller *et al*. 2000; Simons *et al*. 2014). Carotenoid-based colored teguments vary seasonally (Negro *et al*. 1998; Pérez-Rodríguez 2008), as well as in response to changes in nutrient availability (Velando *et al*. 2006; Costantini *et al*. 2007; Pike *et al*, 2007), immune challenges (Faivre *et al*. 2003; McGraw & Ardia 2003; Torres & Velando 2007; Baeta *et al*. 2008) and oxidative threats (Torres & Velando 2007; Alonso-Alvarez & Galván 2011). Accordingly, sexual partners have been found to respond to such dynamic variation by modifying the probability to copulate (Torres & Velando 2003, 2005), the laying date, clutch size (Alonso-Alvarez *et al*. 2012), egg size (Velando *et al*. 2006), and egg composition (Dentressangle *et al*. 2008). Mates' response to variation in carotenoid-dependent colors throughout the reproductive season, suggests that individuals use ornament variation to adjust their investment based on changes in the benefits expected from the current reproductive event. Some studies suggest that carotenoid-dependent colorations are repeatable through time, in spite of being flexible enough to mirror changes in individual condition (Pérez-Rodríguez 2008; Dawson & Bortolotti 2006). However, whether carotenoid-dependent colors can mirror the impact of physiological or environmental changes on individual condition maintaining intra-individual consistency over time remains unclear.

The brown booby (*Sula leucogaster*) is a socially monogamous long-lived seabird with low frequency of extra-pair copulations (Gañán *et al*. 2014), only one reproductive attempt per year and only one survivor offspring per reproductive attempt (Nelson 1978). Brown booby pairs incubate a modal clutch of two eggs during 42 days (± 3 days), and feed the offspring up to five months after hatching (Dorward 1962; Nelson 1978). This species exhibits obligate siblicide, within five days after hatching the older chick regularly eliminates the younger one, even in the absence of resources restriction (Drummond *et al*. 2003; Osorno & Drummond 2003).

Eastern Pacific colonies of brown boobies show sexual dichromatism in their bare skin (i.e., lores, cheek, gular and feet). During courtship males display blue-greenish skin color, whereas females' skin color is yellowish. The timescale in which gular color vaies in males and females brown boobies is not know yet. Sky-pointing, one of the most frequent courtship displays in this species, consists of beak elevations that expose the bare skin under the beak to potential mates (hereafter gular). High performance liquid chromatography of this skin portion has revealed carotenoid deposition in both male and female teguments (Montoya B, Flores, C. and Torres R, Unpublished data). Male gular color during courtship is negatively related to first egg volume, but positively associated with male's nest attending, provisioning, as well as offspring size and mass gain (Montoya & Torres 2015). Female's gular color during courtship is negatively associated with male nest attendance, and positively with female nest attendance and chick begging rate (Montoya & Torres 2015). Although gular color during courtship seems to be an indicator of parental effort, particularly in males, we still do not know how gular color varies throughout the different stages of the reproductive cycle.

Here, using color segments calculated from a model of human visual perception and, photon catch by cones from an average model of avian visual perception with UV sensitivity, we evaluated in a cross-sectional sample whether gular color varied in male and female brown boobies over a reproductive event. Considering that gular color in both sexes is associated with parental effort, and that in the brown booby mutual mate choice is likely (Montoya & Torres 2015), we do not expect differences between sexes in the dynamics of gular color variation though the reproductive season. If color announces readiness to reproduce or if pigments are allocated strategically to color skin when competition for mates is more intense, gular color is expected to increase as laying approaches. Considering that brown booby gular color was recently found to be associated with foraging niche and possibly diet (Michael *et al.* unpublished data), in the case that environmental availability of carotenoids varies as the season advances, we predicted an association between gular color and capture date independent of reproductive activity. Additionally, if color is a signal of reproductive capacity, gular color may be higher in individuals that establish a clutch compared to those which do not. Finally, when comparing intra-individual color variation throughout the reproductive season, we predicted that if gular color has a signaling role mainly during pair formation, it would decline from courtship to hatching and parental care and, if color in this skin area is a consistent within-individual trait, it would be repeatable through the reproductive season.

METHODS

The study was conducted at Isla Larga, Parque Nacional Islas Marietas, Nayarit, Mexico (20º 41'N 105º 36' W) in the Eastern Pacific, during the brown booby breeding season, between June and October, 2011.

During the courtship period (June and July), pairs of birds observed near each other in previously identified courting sites were captured using night lighting (Torres & Velando 2005), marked with white polymethylmethacrylate numbered leg bands (Interrex, Poland) and measured their body mass (± 20 g), ulna length (± 1 mm), and the color of the bare skin under the bill. Once all measurements were completed, roughly within 10 minutes after capture, birds were returned to their courting site that was marked with a numbered flag. Marked nests were visted the day after capture to confirm the identity of the pairs and monitored daily to record laying date. Pairs that established a clutch were recaptured the day the first chick hatched and 15 days after that to obtain new color measurements. From the initial sample, 53 pairs established a clutch and were measured at hatching, and from these, 39 had at least one chick alive and were re-measured fifteen days after the first egg hatched.

Skin color was quantified using a reflectance spectrophotometer (MINOLTA CM 2600d, Osaka, Japan), that measures reflectance at 10 nm intervals from 360 to 740 nm (data from 360 to 700 mn were used in the analyses). This portable device uses diffuse illumination, emitted by 3 internal pulsed xenon lamps set at 10° and received by a silicon photodiode array (dual 40 elements). A target mask (diameter 8 mm) was used to prevent external light entering. Three sequential measurements (3 seconds in between) of non-overlapping segments of the gular skin were taken by placing the spectrophotometer at 90° from the skin surface and changing the position of the apparatus with respect to the initial position in the skin surface. The average of these three measurements is automatically estimated for the apparatus and was used to obtain a reflectance curve for each bird. Color data was downloaded for further analyses using OnColor software (CyberChrome, Inc.). During every measurement session the device was calibrated against a white and a black target. The gular color of male brown boobies displays 2 main peaks, one at 360 and the other at 540 nm, whereas females' gular color peaks at 520 and 620 nm. To analyze the variability along the reflectance spectra we calculated (1) Endler's segments (Endler 1990), and (2) estimated photon catch for each of the four single cone receptors, UV (UWL) = 300 – 410 nm (360 – 410 nm in our dataset), short wavelength (SWL) = 400 – 550 nm, medium wavelength (MWL) = 500 – 620 nm, and long wavelength (LWL) = 550 – 700 nn (Vorobyev & Osorio 1998, Vorobyev *et al.* 1998; Endler *et al*. 2005). To calculate Endler's segments, first we obtained the total brightness as the sum of the reflectance from 360 to 700 nm. Then, we divided the spectrum in four non-overlapping segments, UV = 360 – 400 nm, blue = 410 – 500 nm, green = 510 – 600 nm and red = 610 – 700 nm, which approximately correspond to the four cone sensitivities in birds (Endler & Mielke 2005). Finally, we divided each of these segments between the total brightness obtained in the first step. In general, models of visual perception account for specific sensitivity of the different cones of the visual system studied, and quantify the stimulation provide by a specific color to cones, this by estimating photon catch by each cone (Maia *et al*. 2013). To calculate photon catch we used the function vismodel of R package pavo (Maia *et al*. 2013), specifying the avian average UV as visual system and maintaining other default settings (achromatic stimulation, illuminant and background). We obtained estimates of photon catch for each individual cone receptor. Photon catch estimation assuming an UV sensitive visual system might be affected by the fact that the device we used to measure color in this study obtains reflectance values starting from 360 nm. Consequently, information about reflectance between 300 to 360 nm is unavailable.

Statistical analyses

We used independent lineal mixed models with normal error distribution and bird identity as a random factor to evaluate whether color segments and photon catch differed by sex during each reproductive stage. There were differences between sexes in all color segments at the three reproductive stages (Fig. 1a and b). Thereby, male and female gular color was analyzed in independent equivalent models using as response variables (1) color segments from a human model of visual perception and, (2) photon catch by cone from an average avian model of visual perception with UV sensitivity. General lineal mixed models (GLMM) with normal error distribution were fitted to analyze within subject variation in gular color throughout three reproductive stages (courtship, hatching and parental care). The models included capture date (date when an individual was captured) as an estimate of environmental variation, and bird identity as a random factor.

Individual repeatability of color variables between reproductive stages was calculated using the R package rptR (Nakagawa & Schielzeth 2010). Repeatability estimates were obtained by fitting a GLMM and dividing the between-group variance between the sum of the between-group and the within-group (residual) variance. Parametric bootstrapping was used to obtain confidence intervals for the mixed-model-based repeatability, and statistical significance of the repeatability estimates was tested by restricted maximum likelihood (Nakagawa & Schielzeth 2010). This procedure allowed us to evaluate the proportion of the total between individuals variation that is repeatable within repeated measures of the same subject. Thus, significant repeatabilities indicate that the individual's measurements throughout the reproductive season are more similar within them than compared with other individuals' measurements, as long as there is between individuals variability (Nakagawa & Schielzeth 2010).

General linear mixed and repeated measures models were performed using SAS 9.0 (SAS Institute 1999). All other analyses were carried out using R 2.0.2 (R core team 2014). A backward deletion procedure of non-significant terms was used to fit the minimum adequate models. Residuals from all final models were normally distributed. Means ± standard deviations are shown throughout the manuscript.

RESULTS

Color variation with laying date, reproductive stage and capture date

Gular color at courtship, hatching and parental care differed between males and females (Fig. 1a and b). During courtship, male and female gular color segments did not change as laying approached (interaction between days to lay * sex for brightness $F_{1,89} = 0.57$, $P = 0.45$; UV $F_{1,89} = 0.40$, $P = 0.53$; blue $F_{1,89} = 0.40$, $P = 0.53$; green $F_{1,89} = 0.81$, $P = 0.37$; red $F_{1,89} = 0.10$, $P = 0.75$). Photon catch by cone receptor during courtship did not vary with laying date either in males or females (interaction between days to lay * sex for total photon catch $F_{1,89} = 0.48$, $P = 0.49$; UWL $F_{1,89} = 0.001$, $P = 0.97$; SWL $F_{1,89} = 1.96$, $P = 0.16$; MWL $F_{1,89} = 0.48$, $P = 0.49$; LWL $F_{1,89} = 0.29$, $P = 0.59$).

Gular color segments varied with reproductive stage in both, females and males. Female gular blue at courtship was 6.55% lower than at hatching and 3.76%

than at parental care (Table 1; Fig. 2a). Conversely, female gular green at courtship was 4.5% higher than at hatching and 1.25% than at parental care (Table 1; Fig. 2b). Female gular total brightness, UV and red did not change with the reproductive stage (Table 1). At courtship, male gular brightness, blue and red were 8.33, 6.22 and 5.09% respectively lower than at hatching, and 4.58, 11.10, 9.55% respectively lower than at parental care (Table 1; Fig. 2a - c). Contrastingly, male gular green and UV at courtship were 1.19 and 10.29% respectively higher than at hatching and 2.86 and 12.55% respectively higher than at parental care (Table 1; Fig. 2b - d). Female and male gular green were negatively associated with capture date, irrespectively of the specific reproductive stage (female, $\beta = -0.0005 \pm 0.0002$, male, $\beta = -0.0004 \pm 0.0002$; Table 1). Male gular red was also negatively associated with capture date regardless of the specific reproductive stage ($\beta = -0.0004 \pm 0.0002$; Table 1). There was no association between female or male gular total brightness, UV or blue and capture date, and female gular red (main effects and interaction, Table 1).

When the specific avian visual system was considered (photon catch estimates), female gular variation in the four photon receptors was unrelated to reproductive stage and capture date (main effects and interaction, Table 2). Male gular total and SWL photon catch estimates varied with the reproductive stage (Table 2). Male gular total photon catch at courtship was 7.41% higher than at hatching, and 9.43% higher than at parental care (Table 2). Unlike total photon catch, male gular SWL at courtship was 12.27% lower than at hatching and 14.36% lower than at parental care. Male gular LWL at courtship tended to be 11.89% higher than at hatching and 13.48% than at parental care ($P = 0.08$; Table 2). Only male SWL tended to vary with capture date ($P = 0.056$; $\beta = 0.001 \pm 0.0007$; Table 2), other gular photon catch estimates in males were not associated with reproductive stage (main effects and interaction, Table 2). Male gular photon catch estimates were not associated with capture date (Table 2).

Clutch establishment

Gular color segments did not differ between females that established a clutch and those that did not (brightness $F_{1,83} = 1.51$, $P = 0.22$; UV $F_{1,83} = 0.001$, $P = 0.98$; blue $F_{1,83} = 0.04$, $P = 0.84$; green $F_{1,83} = 0.02$, $P = 0.88$, and red $F_{1,83} = 0.09$, $P = 0.76$). However, when the visual system was considered, female gular total and MWL photon catch were 11.38 and 22.76% higher in individuals that established a clutch compared with those that did not (total photon catch $F_{1,82} = 4.69$, $P = 0.03$; MWL $F_{1,82} = 6.79$, $P = 0.01$; Fig 3a and b). Female gular photon catch by other receptors did not differ between individuals that established a clutch and those that did not (UWL $F_{1,83} = 0.56$, $P = 0.46$; SWL $F_{1,83} = 2.49$, $P = 0.12$; and LWS $F_{1,83} = 0.04$, $P = 0.84$).

Male gular color segments did not differ between individuals that established a clutch and those that did not (brightness $F_{1,94} = 0.95$, P = 0.33; UV $F_{1,83} = 0.10$, $P = 0.76$; blue $F_{1,83} = 0.23$, $P = 0.63$; green $F_{1,83} = 0.03$, $P = 0.86$, and red $F_{1,83} = 1.04$, $P = 0.31$). However, when the visual system was considered, gular of males that established a clutch had 18.26% higher LWL photon catch compared with males that did not ($F_{1,93} = 4.28$, $P = 0.04$; Fig 3c). Gular total, UWL, SWL and MWL photon catch did not differ between males that established a clutch and those that did not (total photon catch $F_{1,94} = 0.34$, $P = 0.56$; UWL $F_{1,94} = 0.38$, $P = 0.54$; SWL $F_{1,94} = 0.17$, $P = 0.68$; and MWL $F_{1,94} = 0.18$, P=0.67).

Repeatability

Repeatability through the reproductive season (i.e. courtship, hatching and parental care) of gular brightness was 40% in females and 47% in males (Table 3). Female gular UV and green were 59% and 54% respectively, repeatable throughout the reproductive season (Table 3). Male gular color segments and female blue and red were all non-repeatable (Table 3).

Female gular total and specific cone photon catch were unrepeatable throughout the reproductive season, while male gular total photon catch was only marginally 36% repeatable (Table 3). Male gular photon catch by specific visual receptor was non-repeatable (Table 3).

DISCUSSION

In the brown booby, gular color differed by sex through the reproductive season. Color in males and females changed with date and reproductive stage, but not with laying proximity, and female color showed higher repeatability throughout the reproductive season than male color. When an avian visual model with UV sensitivity was used, no changes in female photon catch associated with laying proximity, capture date, or reproductive stage were identified, yet females that laid eggs had higher photon catch. In males, gular photon catch varied with reproductive stage, marginally with capture date and, between males that established a clutch and those that did not. Repeatability of gular photon catch was only marginally significant in males, while absent in females. Overall, results suggest that although male and female skin colors differ, similar variation through the season in the blue and green color segments, potentially linked to the deposition of carotenoid pigments, might imply similar mechanisms of color production. Also, gular color variation may have a role as an informative signal in males and females, because color varied in relation to variables potentially linked to sexual selection, and based on the results obtained when the specific avian visual model was considered, this variation between and within individuals appeared to be perceivable (at least at some wavelengths) by conspecifics.

In males and females, gular green and blue varied over the reproductive season following a similar pattern. Male and female gular green was higher during courtship than afterwards, whereas blue was lower in courtship than in subsequent stages. The pattern of color variation from courtship to incubation and parental care in these color segments is consistent with the idea of a higher deposition of carotenoid pigments during courtship. Carotenoid pigments reflect light after 500 nm, resulting in higher green reflectance, and absorb at shorter wavelengths, resulting in lower blue reflectance (McGraw 2006). Accordingly, the presence of carotenoids on skin samples collected at the courting stage from 4 males and 2 females was determined (Montoya, B., Flores C. and Torres, R. unpublished data). In the sister species, the blue footed booby (*Sula nebouxii*), the blue turquoise foot color displayed during courtship can change in a short period of time (24 to 48 hours) after a carotenoid supplementation, suggesting a likely interaction between the collagen structure of the skin and the deposition of yellow carotenoids (Velando *et al.* 2006, Torres & Velando 2010). In brown boobies, the variation found in gular blue and green segments may rely on a similar mechanism than that suggested for the blue-footed booby, if this hypothesis is true, the mechanism behind skin color production in this *Sulidae* family might be phylogenetically conserved (Torres & Velando 2010). Interestingly, only in males, gular UV was higher during courtship than afterwards.

Male gular UV might interact with green and blue to produce a specific visual stimulation, as has been suggested for other carotenoid-dependent colored teguments (Prum & Torres 2003; Mougeot *et al.* 2007). From a previous study, we know that male gular green and UV during courtship are negative correlated (Montoya & Torres 2015), albeit reflectance in both segments is higher during courtship than afterwards, suggesting that UV and green reflectance might interact in the production of a color signal used during mate choice. The results suggest that despite the conspicuous difference in gular color by sex, the mechanism of color production in brown booby males and females might be similar.

In addition to variation between reproductive stages, male and female gular green decreased with capture date. Similar variation between sexes in green components of the gular color through reproductive stages suggests that color production is costly and is used by both sexes as a signal with informative value only during courtship. At courtship, gular green in males was 2.86% higher than at parental care, while in females it was 1.25% higher. On the other hand, a decline with date in gular green color might result from variation in environmental availability of carotenoids affecting both sexes. Interestingly, the magnitude of green color variation with capture date differed by sex, 2.94 and 11.11% for males and females, respectively. In the brown booby, a recent study showed that male gular green is related to better and farther foraging niches and females that exploit similar type of niches are paired with males with greener gulars (Michael, N. and cols. unpublished data). Moreover, previous studies have shown that brown booby females exploit farther foraging niches and have longer foraging trips than males (Weimerskirch *et al.* 2009). In our study, capture date is probably related to the individual's time of arrival to the courtship site, and individuals arriving late in the season are likely to be of lower quality (e.g. Morbey & Ydenberg 2000; Christians *et al.* 2001). Additionally, later in the breeding season food availability and overall breeding conditions might deteriorate (e.g. Verhulst *et al.* 1995; Verboven & Visser 1998; Dubiec & Cichoń 2005). Hence, the results suggest that gular color might have a role as a sexual signal during courtship, when probably a greater allocation of carotenoid pigments to color the gular skin is favored. Environmental variation in carotenoid availability may explain the decline in gular green with date; however, differences in individual quality and foraging range by sex, may explain the disparity in the decline with date in gular color of males and females.

As light spectrum that reaches the eye depends on both, reflectance spectrum and ambient light spectrum (Endler 1990), it is important to acknowledge that our measurements of color change comprise variation only in reflectance spectrum. However, ambient light could also have changed throughout the reproductive season affecting estimation of color variation and repeatability. Furthermore, in our study photon catch estimation could have been affected by the absence of information of light reflectance between 300 and 360 nm. Thereby, results from visual models need to be taken cautiously. When the visual system was considered, in males, total photon catch and short wavelength stimulation varied with the reproductive stage, whereas in females no significant change in photon capture associated with reproductive stage was found. These results suggest that, unlike variation in female gular color, variation over the reproductive event in male gular total brightness, blue and part of the green segment might be perceived by other brown boobies; a necessary condition for gular color to function as a signal.

In females, the magnitude of change in the blue and green segments, from courtship to parental care, was smaller than found in males. Consequently, within-individual variation through reproductive stages in female gular color may not be large enough to be perceivable. Interestingly, gular color of males and females at courtship differed between individuals that later established a clutch and those that did not do so. Females that laid eggs had gulars with higher total and medium wavelength photon catch, whereas gular of males that established a clutch had higher long wavelength photon reception. In males and females, gular photon reception during courtship might be associated with other fitness traits that contribute to successful clutch establishment. Males and females that established a clutch might have been in better condition, since gular color in both sexes is carotenoid dependent (Montoya, B., Flores, C. and Torres, R. unpublished data), and in males is associated with better and farther foraging niches (Michael, N. and cols. unpublished data). Therefore, individuals with lower gular photon catch might have constrained reproductive investment privileging self-maintenance because of their poorer nutritional and consequently general condition. Hence, in males within-individual variation in gular color through different stages of the reproductive event might have an informative value to con-specifics, and in males and females between individuals' differences in gular color at courtship might have a signaling role.

In females, gular UV, total brightness and green were above 40% repeatable throughout the reproductive event. Gular UV did not differ statistically among reproductive stages and was highly repeatable, suggesting that female gular UV is an individual characteristic that remains relatively constant over time. Conversely, female total brightness and green differed statistically among reproductive stages. Thereby, the moderate repeatability found in these parameters suggests that despite variation over time, gular brightness and green are more within individual females among stages, than between all the females sampled. In males, only total brightness was repeatable throughout the reproductive season, 47%, and this parameter also changed statistically among reproductive stages. Therefore, despite changes found, gular brightness appears to remain more similar within individual males over time, than between different males. When visual system was considered female gular color resulted unrepeatable throughout the reproductive season. Interestingly, only the total photon reception of male gular was repeatable (36%, marginal significance $P = 0.056$), suggesting that other brown boobies could perceive that variation in male gular total photon catch, over the reproductive attempt, are more within individuals than between them. The fact that some components of gular coloration in both, males and females are repeatable through time suggests that if gular color is condition-dependent, then individuals with better gular color also maintain better condition through the reproductive season.

In conclusion, our results suggest that the mechanism of color production in males and females might be similar. However, breeding decisions in terms of how much to invest in ornaments probably differ for males and females. Variation in gular color is probably linked to environmental fluctuation through the reproductive season in the availability of carotenoid pigments; yet differences in decisions on ornament investment, foraging ability or individual quality might explain the difference by sex in color variation through the season. Interestingly, when the visual model was used, males and females that eventually established a clutch were significantly more colorful from those that failed to reproduce, and these differences were presumably detectable by con-specifics. Thus, in the brown booby male and female gular color is a dynamic and probably condition-dependent trait, with certain level of within-individual repeatability through the breeding season. Patterns of variation in gular color suggest that within-individual color variation in males and, between-individuals in males and females might have informative value to other members of the species.

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Table 1. Female and male color changes by segment in relation to reproductive stage and date.

	T. brightness		UV		Blue		Green		Red	
Females	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Stage	2.91	0.09	0.52	0.47	**10.53**	**0.001**	**4.09**	**0.04**	2.79	0.10
Date	1.71	0.19	0.31	0.58	0.84	0.36	**4.79**	**0.03**	0.19	0.66
Stage * date	0.23	0.63	0.62	0.43	1.30	0.26	2.70	0.10	0.10	0.75
Conditional R^2	-		-		0.35		0.26		-	
Error df final model	153		153		152		151		152	
Males										
Stage	**15.05**	**<0.001**	**45.45**	**<0.001**	**93.19**	**<0.001**	**5.74**	**0.02**	**8.99**	**0.003**
Date	1.18	0.28	2.04	0.16	7.59	0.007	**6.51**	**0.01**	**3.85**	**0.051**
Stage * date	0.02	0.88	1.58	0.21	0.26	0.61	0.91	0.34	0.17	0.68
Conditional R^2	0.49		0.58		0.56		0.36		0.52	
Error df final model	166		166		165		165		165	

Independent linear mixed models were fitted by sex with bird identity as a random factor. In bolds *F* and *P* values of the final models. Sample size at courtship was 84 females and 95 males; at hatching 36 females and 38 males; and at parental care 36 females and 36 males. Conditional R^2 (i.e. the proportion of variance explained by the fixed and random component) was calculated for final models.

Table 2. Female and male color changes by photon catch receptor in relation to reproductive stage and date of capture.

	Total photon		**UWL**		**SWL**		**MWL**		**LWL**	
Females	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Stage	0.54	0.47	0.72	0.40	0.60	0.44	0.04	0.84	0.05	0.82
Date	0.23	0.63	0.02	0.89	0.08	0.78	0.05	0.83	0.09	0.77
Stage * date	0.03	0.85	0.34	056	0.28	0.60	0.01	0.92	0.38	0.54
Error df final model	153		153		153		153		153	
Males										
Stage	**5.95**	**0.02**	0.001	0.99	**5.89**	**0.02**	0.23	0.63	3.16	0.08
Date	1.81	0.18	1.76	0.19	**3.71**	**0.056**	0.02	0.89	1.53	0.22
Stage * date	0.17	0.68	0.01	0.93	0.40	0.53	0.12	0.73	0.03	0.85
Conditional R^2	0.33		-		0.12		-		-	
Error df final model	165		166		165		1.66		166	

Independent linear mixed models were fitted by sex with bird identity a random factor. In bolds *F* and *P* values of the final models. Sample size at courtship was 84 females and 95 males; at hatching 36 females and 38 males; and at parental care 36 females and 36 males. Conditional R^2 (i.e. proportion of variance explained by the fixed and random component) was calculated for final models.

Table 3. Repeatability of color measurements between courtship, incubation and parental care.

	Females				**Males**			
	R	**Lower CI**	**Upper CI**	***P***	***R***	**Lower CI**	**Upper CI**	***P***
Bright	**0.40**	**0.09**	**0.64**	**<0.001**	**0.47**	**0.24**	**0.66**	**<0.001**
UV	**0.59**	**0.28**	**0.77**	**0.002**	0.18	0.00	0.41	0.99
Blue	0.43	0.10	0.67	0.99	0.12	0.00	0.36	0.99
Green	**0.54**	**0.24**	**0.74**	**0.01**	0.27	0.05	0.49	0.99
Red	0.42	0.11	0.66	0.99	0.29	0.04	0.50	0.99
T. photon	0.00	0.00	0.28	0.99	**0.36**	**0.10**	**0.57**	**0.056**
UWL	0.04	0.00	0.31	0.99	0.37	0.10	0.56	0.09
SWL	0.38	0.05	0.61	0.31	0.07	0.00	0.31	0.99
MWL	0.00	0.05	0.30	0.99	0.22	0.00	0.45	0.99
LWL	0.00	0.00	0.22	0.99	0.26	0.003	0.49	0.99

Linear mixed models were fitted by sex (males n = 28, females n = 19). Repeatability estimates were obtained by dividing the between-group variance between the addition of the between-group and the within-group (residual) variance. Parametric bootstrapping was used to obtain confidence intervals, and restricted maximum likelihood to test statistical significance.

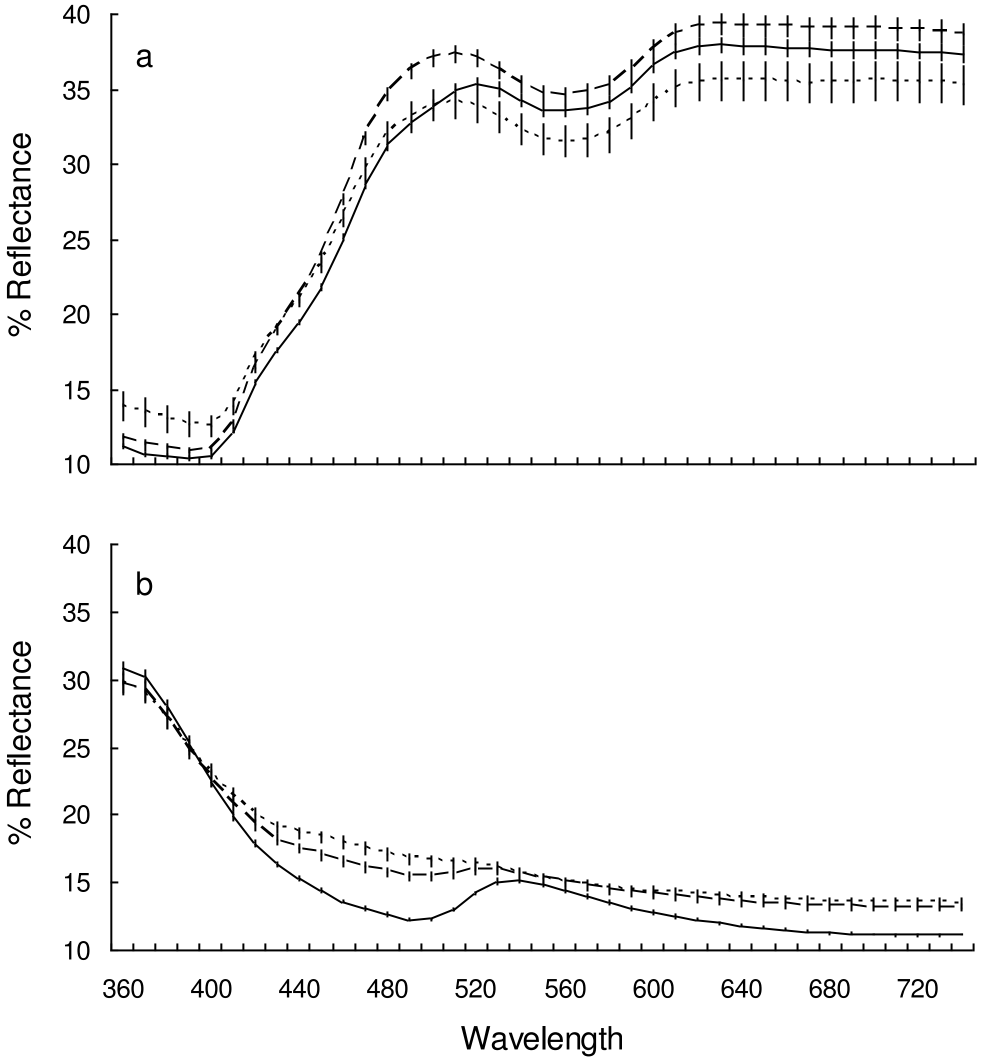


Figure 1. Gular skin reflectance in (a) females and (b) males. In both figures, continue line represents reflectance values at courtship, dotted line at hatching and dashed line at parental care. Figures show average reflectance spectra (± SE) of 95 males and 84 females in courtship, 38 males and 35 females in hatching, and 36 males and 35 females in parental care.

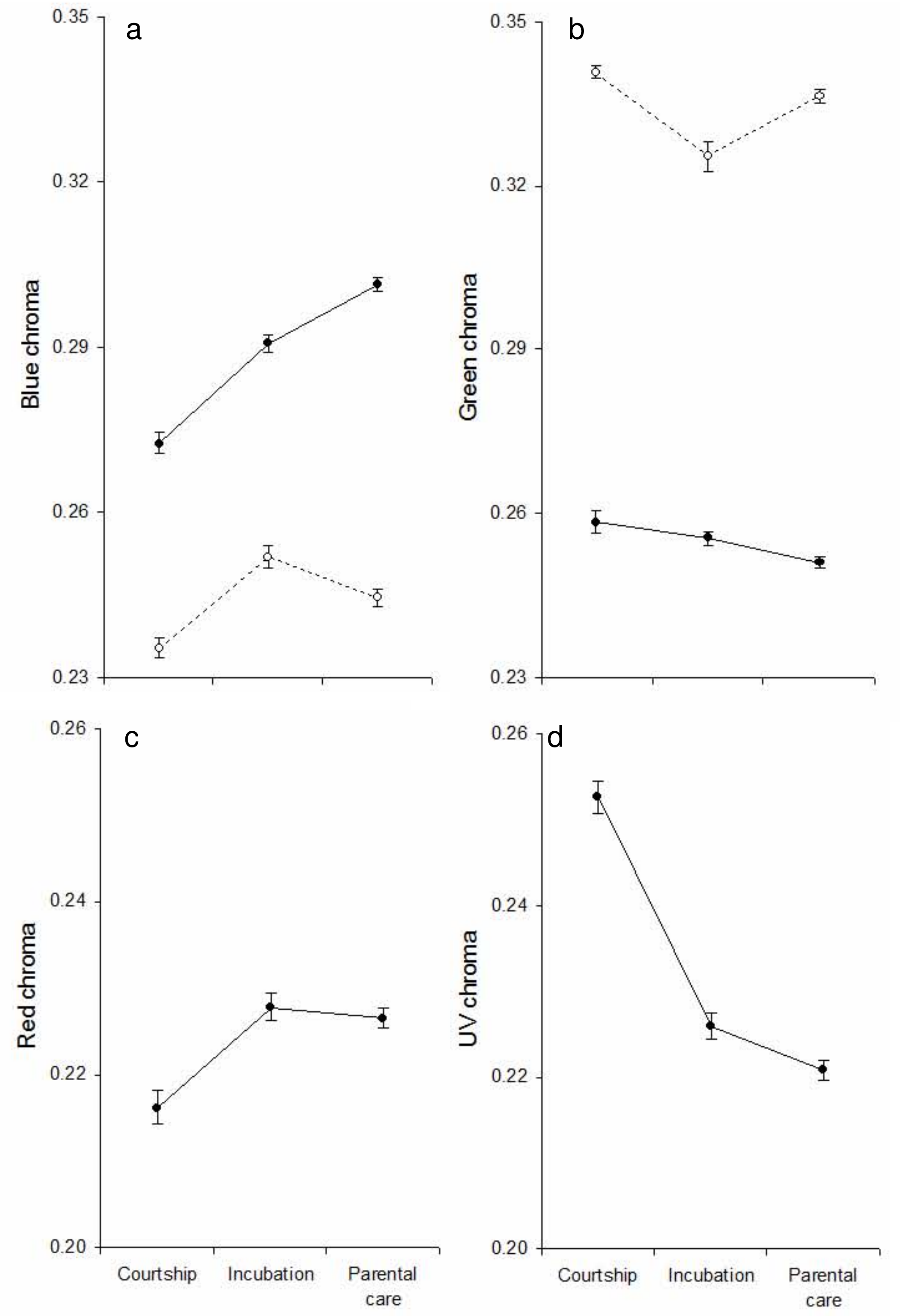


Figure 2. Change in gular color throughout the reproductive season. Males are represented by closed dots and solid lines and in females by open dots and dashed lines. Panels correspond to chromas (a) blue, (b) green, (c) red, and (d) UV. Values represented are mean ± SE.

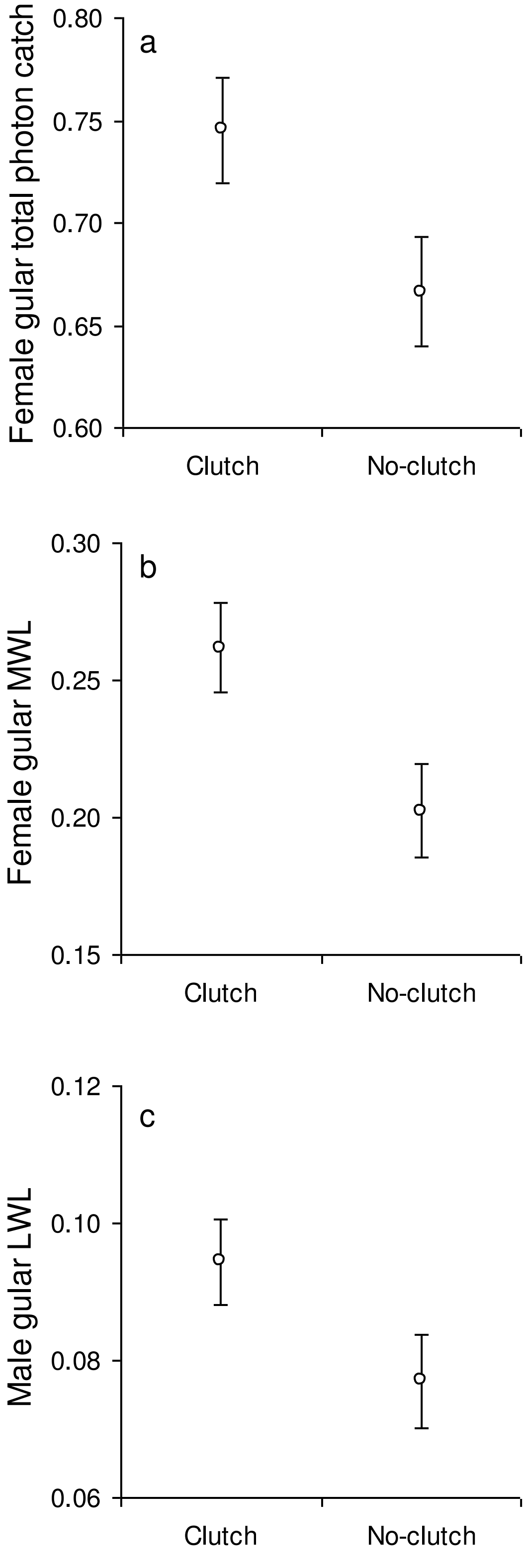
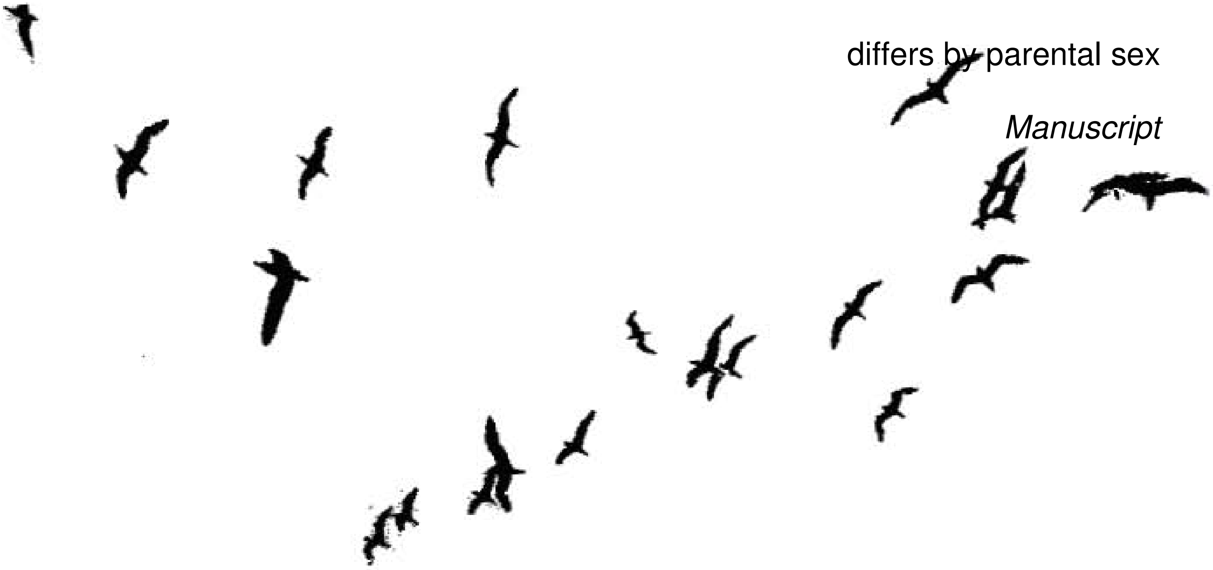


Figure 3. Difference between individuals that established a clutch and those which did not. (a) Female gular total photon catch, (b) female gular MWL and (c) male gular LWL. Values represented are mean ± SE.

CAPÍTULO V

Parent offspring co-adaptation in an obligated bi-parental care species differs by parental sex

Manuscript



Parent offspring co-adaptation in an obligated biparental care species differs by parental sex

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Running title: Differential parent-offspring co-adaptation by parental sex

ABSTRACT

Parental supply and offspring demand during parental care are non-independent behaviors. The association between the two has been proposed to lead to co-adaptation through selective pressures favoring specific combinations above others. Consequently, costs are expected to arise when co-adaptation is disrupted. We investigated the occurrence of a correlation between genetic parents provisioning and their genetic offspring begging, and the consequences of disrupting this correlation on the immune response of family members and the offspring body mass gain. We used a clutch cross-fostering experiment to separate genetic and maternal effects from rearing parents' effects on the studied correlation. In both control and cross-fostered group, we found a positive correlation between provisioning by rearing parents and their dependent offspring's begging. When chicks were reared by foster parents, there was a negative correlation between genetic mother provisioning and genetic offspring demand, but no correlation was found between fathers and their genetic offspring. Disrupting the correlation between genetic mothers and their genetic offspring by cross-fostering clutches, affected negatively immune response of fathers, and probably of mothers. Our study suggests that in the brown booby there might be a negative mother-offspring co-adaptation, probably favored by selective pressures on mother provisioning. Future studies should investigate whether this negative correlation between mother provisioning and offspring demand is a direct genetic effect or a maternal effect, and the fitness consequences of this presumably co-adaptation.

Key words: Interacting phenotypes, parental care, offspring begging, conflict, co-adaptation, family dynamics, *Sula leucogaster*.

Reproductive investment can entail costs in two levels: through a reduction in the parents' survival and probability of future reproduction and through a reduction in the offspring fitness (Stearns 1989). These costs are the engine of the parent-offspring conflict, which is determined by the fact that the optimal level of parental investment differs between the parents and their offspring (Trivers 1974). Thus, the level of parental investment that improves offspring fitness may in turn deteriorate parents' reproductive prospective (Trivers 1974). Signals are proposed to mediate the communication in the interaction between family members over parental care, and they need to reliably indicate offspring condition to modulate parental effort (Grafen 1991; Johnstone 1999). Inter-individual variation in the expression of offspring demand signals and parental provisioning was traditionally thought to depend on environmental factors, yet an increasing number of studies have shown variation in both traits to depend also on parent of origin factors (Kölliker *et al*. 2000; Agrawal *et al*. 2001; Kölliker & Richner 2001; Dor & Lotem 2009).

Behavioral interactions within the family result in a co-variation in the expression of two traits: parental supply and offspring demand. This co-variation leads to a form of inheritance where parents provide to offspring both, genes and environment that influence offspring's begging behavior, and offspring provide an environment that can affect parents' expression of provisioning (Wolf & Brodie 1998; Wolf 2000; Kölliker *et al*. 2005; Kölliker *et al*. 2012). Hence, the expression of parental supply might be influenced by genes expressed in two genomes: genetic variation in parental predisposition to provision and genetic variation in offspring solicitation (Kölliker *et al*. 2000; Agrawal *et al*. 2001). Consequently, offspring's traits are under the selective pressures set by the parental care environment, and parents' traits under the selective pressures established by the offspring demand, besides the fitness benefits parents can obtain from investment (Kölliker *et al*. 2012). A Co-adaptation between parental and offspring traits might evolve if particular combinations of supply and demand have on average higher fitness than random combinations or individual traits (Phillips & Arnold 1989; Wolf & Brodie 1998). In such a case, the co-adaptation model of parent-offspring interactions predicts that a disruption of the favored combinations (i.e. co-adapted traits) would impose costs on the parents or the offspring. Which member of the interaction could mainly pay the costs of the disruption, would depend on the original direction of the correlation and thereby, on which trait is under stronger selective pressure (i.e. parental or offspring trait; Kölliker *et al*. 2005). The occurrence of such costs has only been explored recently (Kölliker *et al*. 2005; see Hinde *et al*. 2010; Estramil *et al*. 2013).

Experimental studies regarding the co-adaptation between parental supply-offspring demand have found differences in the direction of the genetic correlation between these two traits. In some species, a positive genetic parent-offspring correlation has been reported (i.e. genetic parents with higher predisposition to provision have genetic offspring with higher predisposition to demand; Kölliker *et al*. 2000; Lock *et al*. 2004; Hinde *et al*. 2009), while a negative correlation has been found in other studies (i.e. genetic parents with higher predisposition to provision have genetic offspring with lower predisposition to demand; Agrawal *et al*. 2001; Maestripieri 2004). Kölliker *et al*. (2005) using a quantitative genetic approach proposed a co-adaptation model to investigate the evolutionary maintenance of parent-offspring interactions. The model proposes that the direction of the correlation between genetic parents' supply and offspring demand depends on two factors: the proximate mechanisms regulating the interaction, and the relative strength of selection on parental and offspring traits (Kölliker *et al*. 2005). According to the model, a positive correlation between genetic parents' and offspring' traits is expected when at the phenotypic level parental provisioning diminishes offspring solicitation and offspring begging is costly, so parents control provisioning and solicitation is under stronger selective pressure (Kölliker *et al*. 2005). In such a scenario, only offspring of very good providers can afford to demand at high levels. In contrast, a negative correlation is expected between genetic parent's and offspring's traits, when at the phenotypic level parental provisioning increases as offspring solicitation augment and begging entails low costs, so offspring have more control on parental provisioning, exerting stronger pressure on this trait (Kölliker *et al*. 2005). Under this condition, combinations of good parental suppliers with less demanding offspring would be more efficient, because they diminish the overall costs of parental investment. Similarly, combinations of high demanding offspring with low provisioning parents would ensure adequate food supply (Wolf & Brodie 1998; Agrawal *et al*. 2001; Kölliker *et al*. 2005; Hinde *et al*. 2010). Whether the selective pressure is stronger on parental care or offspring elicitation depends on the ecology and life history of the species. For instance, short-lived species with direct sibling rivalry, or species where environmental distribution of resources limits parental provisioning are proposed to show a stronger pressure on offspring demand, leading to a positive correlation between genetic parents' provisioning and offspring begging (Kölliker *et al*. 2005). Unlike the former ones, long-lived species with only a single offspring per reproductive attempt, or short-lived species that provision the entire brood as a whole are expected to deal with a stronger selective pressure on parental supply, driving a negative correlation between genetic parents and offspring traits (Agrawal *et al*. 2001; Kölliker *et al*. 2005).

The brown booby (*Sula leucogaster*) is a long-lived sea bird, with an extended period of obligate biparental care. Both parents incubate the clutch during 42 days (± 3 days), and rear and feed the chicks during about 5 months, until the chicks fledge and reach independence (Dorward 1962; Nelson 1978). On average, males spend less time attending the offspring than females, yet there is not difference in provisioning behavior between sexes (Montoya & Torres 2015). As main mortality by predators occurs during the egg and early-life stages, neither eggs nor young chicks can be left unattended (Nelson 1978). Brown boobies lay a modal clutch size of two eggs (mean ± SE: 1.88 ± 0.05, in our sample); but typically, only one chick survives until fledging (0.92 ± 0.04, in our sample; see also Nelson 1978). Brood reduction occurs because soon after hatching the older chick regularly attacks and eventually expulses from the nest the younger one, even when food appears to be enough to feed both chicks (Drummond *et al*. 2003; Osorno & Drummond 2003). During the first three months of the chick's life, the mother and the father provide food several times a day by transferring small portions of semi-digested fish directly into the chick's mouth (Nelson 1978; Tershy & Croll 2000). Both parents increase food delivery in response to more frequent offspring begging calls, and chick body mass when 15 days old is positively associated with maternal and paternal provisioning rates (Montoya & Torres 2015). Male's food provisioning is signaled during courtship through sexual ornaments (Montoya & Torres 2015). Males with greener gular skin provide more food to the offspring, and have heavier and larger chicks (Montoya & Torres 2015). According to theory, for a species as the brown booby, with only one offspring produced per breeding attempt and obligate biparental care, we might expect that offspring would exert a higher control on maternal and paternal provisioning than provisioning would exert on offspring begging behavior leading to a negative correlation between genetic parents supply-offspring demand (Kölliker *et al.* 2005). This suggestion is partially supported by our previous finding that mothers caring for foster chicks increase food provisioning (Montoya & Torres 2015).

We used a reciprocal clutch cross-fostering experiment to investigate the occurrence of a potential co-adaptation between parental supply and offspring demand in the brown booby. Note that such experimental design does not allow separating genetic from maternal effects. Considering the life-history traits of the brown booby, and the positive correlation between rearing parents provisioning and offspring solicitation (i.e. parents increase food provisioning when rearing higher demanding offspring) previously found (Montoya & Torres 2015), we predict that if there is co-adaptation between parental provisioning and offspring begging, there would be a negative correlation between genetic parents provisioning and genetic offspring begging (i.e. parents good at provisioning having low demanding genetic offspring). Because in brown boobies, maternal and paternal investment influences offspring condition (Montoya & Torres 2015), we expect the correlation to occur for both, genetic mother and father provisioning with offspring demand. Additionally, if a co-adaptation between parental provisioning and offspring begging is found, we expect the cross-fostering treatment to cause a disruption of this co-adaptation, increasing the cost of parental effort. Reproductive effort has been shown to impact immune response capacity, a key self-maintenance component (Gustafsson *et al.* 1994; Deerenberg *et al.* 1997), while offspring condition during development is expected to influence chick survival and fitness prospects (De Kogel *et al.* 1997; van de Pol *et al.* 2006; Reid *et al.* 2010; Boonekamp *et al.* 2014). Hence, as indicators of costs we predict that the cross-fostering will negative impact immune response of parents and/or offspring (i.e. lower agglutination or cellular lysis response) and/or body mass gain in offspring. Finally, considering that offspring provisioning does not differ by parental sex in this species (Montoya & Torres 2015), we do not anticipate either the direction of the genetic correlation or the impact of cross-fostering treatment on immune response to differ by parental sex.

METHODS

The study was carried out from June to October 2011 in the brown booby breeding colony nesting at Isla Larga, Parque Nacional Islas Marietas, Nayarit, Mexico (20º 41'N 105º 36' W). In the courtship period (June and July), we observed and identified courting sites during day-light hours, returned to the courting sites at night, opportunistically captured pairs of individuals that were near each other using night-lighting and returned the day after the capture to confirm that captured birds were actual courting couples (Torres & Velando 2005). Fifty-two pairs were captured and within roughly 10 minutes, each captured pair was individually marked with a numbered polymethylmethacrylate leg band (PMMA; Interrex, Poland), blood sampled, and their body mass (± 20 gr), ulna length (± 1 mm) and gular color were measured. Gular color was measured using a portable spectrophotometer that determines the reflectance from 360 to 740 (± 10) nm (MINOLTA CM 2600d, Osaka, Japan). Three sequential measurements of non-overlapping patches from the gular skin roughly 1 cm apart were taken to obtain a mean reflectance curve. To measure immune response, a 3 mL blood sample was taken from the brachial vein. Blood samples were transported on ice until they were centrifuged at 10,000 g for 10 minutes, stored for a month in liquid nitrogen, and finally at -80°C until analyses were performed. Birds were returned to their nest site, which was marked with a numbered flag. Nests were visited daily between 18:00 h and 20:00 h to determine the laying date.

Cross-fostering

We reciprocally cross-fostered 34 complete clutches to separate genetic from phenotypic sources of co-variation between parental effort and offspring demand. However, this experimental design does not control for potential pre-hatching maternal effects, which have found to promote a mother-offspring co-adaptation in other birds (Hinde *et al.* 2009). As control for the cross-fostering procedure, 18 clutches were mock-fostered. Fifteen days after the first egg of the clutch was laid, clutches were randomly assigned to the cross-fostered (n = 28 two-egg and 6 one-egg clutches) or control group (n = 12 two-egg and 6 one-egg clutches). Cross-fostered clutches were matched by laying date (± 3 days) and clutch size (1 or 2 eggs). Because we were also interested, for a different study, on investigating whether male gular color during courtship was an indicator of direct and indirect benefits for females (see Montoya and Torres 2015), we selectively paired clutches whose males had the greatest differences in the gular color at 540 nm (the peak wavelength within the range of green reflectance in our sample) within the nests available (male gular color between males of swapped clutches, $r_p = -0.50$, $P = 0.008$). This non-random pairing of the swapped clutches could confound the interpretation of results, because male gular color was found to be positively associated with male provisioning and negatively with first egg volume, though it is not directly related to mother provisioning or offspring begging (Montoya & Torres 2015). Hence, to control for this potential confounding effect from the experimental design, the direction of the swapping treatment (i.e. whether chicks were swapped from a nest with a colorful father to a nest with a dull male or vice versa) was included in the statistical models when a correlation between genetic parents' variables and genetic offspring's variables was identified.

As hatching date approached and until 15 days post-hatching, nests were visited daily to record hatching date and chick survival. Offspring ulna length (± 1 mm) and body mass (± 1 g) were measured at hatching and every 5 days until chicks were 15 days old. On day fifteen post-hatching, a new 3 mL blood sample was taken from adults and a 1 mL blood sample from chicks. Samples were handled as described above. From the initial sample of 52 clutches, 5 nests had no hatchlings and in 8 nests chicks died before age 15 d. Hence, final sample for analyses consisted of 39 nests, 36 first chicks, and 3 second chicks.

Behavioral observations

When chicks were 1, 5, 10 and 15 days old, parental provisioning (when a parent places the bill open above the chick's head and the chick places its head into the parent's bill), chick begging (when the chick raises the head and vocalizes with a "tac-tac" sound), and the time the male and the female were present on the nest (arrivals to and departures from the nest by each parent) were recorded from 07:00 - 09:00 h and from 17:00 - 21:00 h, by four trained observers seated at 3 - 6 m from the focal nest (detailed descriptions of behaviors in Nelson 1978). Observers recorded absolute occurrence of provisioning by the male or the female, and chick begging to the male or the female in every 5 min intervals (1 - 0 records). Inter-observer reliability was ≥ 90%. Observers were blind about the group to which the focal nest belonged to (cross-fostered or control). For statistical analyses, provisioning by the male or the female (the sum of total occurrence of provisioning events by the male or the female during the four observation periods), and the chick's begging rate to the male or the female (the sum of total begging events to the male or the female during the four observation periods divided by the sum of total time the male or the female was present at the nest) were calculated.

Immune response

As an estimate of the humoral immune response we quantified the levels of natural antibodies (Nabs) and complement cascade using the hemolysis-hemagglutination assay (Matson *et al.* 2005). The hemagglutination component of the assay evaluates the interaction between Nabs and antigens resulting in blood clumping, as Nabs mark exogenous microorganisms to be destroyed by phagocytes. The hemolysis component of the assay targets hemoglobulin released after the lysis of exogenous microorganisms. The exogenous microorganism we used in the assay was rabbit blood cells conserved in Alsever (Dibio 1602 FC). Before the assay, rabbit whole blood was centrifuged for 5 min at 2000 g and supernatant was discharged. Phosphate buffered saline solution (PBS) was added, the suspension was centrifuged for 5 min at 2000 g and the supernatant was removed. This procedure was repeated three times. Hematocrit of the final blood concentration was measured to be 1% diluted in PBS.

For the assay, we placed 25 µl of each individual's plasma sample in six of the eight wells of the first row of a 96-well polysterene plate (Corning Costar #3795, Corning, NY, USA; 8 columns by 12 rows), starting in the second well. 25 µl of sterile phosphate solution (PBS) was placed in the first well as negative control and 25 µl of rabbit whole blood was added to the last well as positive control. After that, we diluted all samples and controls placed in the plate in a range of 1 to 1/1025 by adding PBS through a set of 1:2 serial dilutions. Finally, 25 µl of 1% rabbit blood cell suspension was added to each well, and the plate was sealed with a polystyrene plate lid. Before incubating the plate at 37°C for 90 min (1602H Hova-Bater incubator), it was vortexed for 10 s at a low speed. Once incubated, plates were inclined at a 45° angle along their long axis for 20 min at room temperature. Then, plates were left in a horizontal surface during 70 min at room temperature. Plates were scanned (HP deskjet F4180) at full-size image (300 d.p.i.) right after (i) incubation, (ii) room temperature inclined rest, and (iii) room temperature horizontal rest. Two trained observers independently quantified hemagglutination and complement-mediated lysis by assessing the dilution stage (on a scale from 0 to 11) at which these two reactions stopped (for further details, see Matson *et al.* 2005). Inter-observer reliability was on average 90% for agglutination and 99% for lysis. Similarly, intra-observer reliability was on average 96% for agglutination and 99% for lysis. Repeatability between plates was r = 0.84 for agglutination and r = 0.90 for lysis. The average value between the scores given by the two observers for agglutination and lysis was used for statistical analyses.

Statistical analyses

Analyses were carried out using R 2.0.2 (R core team 2014) and SAS 9.0 (SAS Institute 1999). Minimum adequate models were obtained using backward deletion procedure of non-significant terms (α = 0.05). Eta-squared was used to calculate effect size for principal results (package “lsr”, Navarro, 2015), and results were included in the figure legend and the main text. Three models were tested to evaluate the association between parental provisioning and chick’s begging rate when reared by either genetic or foster parents. Models one and two were fitted using generalized linear models with negative binomial error distribution, link log and *F* tests, to account for over dispersion (Crawley 2002; ver Hoef & Boveng 2007). For model three, a general linear model with normal error distribution was used. The first model included provisioning by the rearing parent as a response variable, and as explanatory variables the swapping treatment (cross-fostered or control) as fixed factor, begging rate by the reared chick, and the interaction between swapping treatment and reared chick begging. The second model included provisioning by the genetic parent as a response variable, and as explanatory variables the swapping treatment as fixed factor, begging rate by the genetic chick, and the interaction between swapping treatment and genetic chick begging. To further investigate a potential genetic correlation, a third model was fitted using only the swapped nests. Only in the swapped nests, to control for the influence of the reared chick begging on parental provisioning, provisioning by the genetic parent was divided by the reared chick begging (hereafter, weighed rate of provision). Similarly, to control for provisioning by rearing parents on chick begging activity, the genetic chick begging rate was divided by the provisioning received by the rearing parents (hereafter, weighed begging rate). The model included the weighed rate of provision as the response variable, and as explanatory variables the weighed rate of provisioning and the specific direction of the swapping treatment (i.e. whether chicks were swapped from a nest with a colorful father to a nest with a dull male or vice versa). In the three models egg volume and laying date were included as covariates to control for a potential effect of mother of origin condition and environmental variation respectively. Independent models by parental sex were adjusted to investigate the relationships between females and males provisioning and offspring begging.

To evaluate potential effects of a disruption of the parent-offspring co-adaptation on immune response of mother and father, we used repeated measures models with normal error distribution. Independent models for males and females were run. The models included as response variables our estimates of immune response (cellular lysis or agglutination), the reproductive stage (courtship or parental care) as within-group fixed factor, the difference in begging rate between focal and genetic offspring as covariate and swapping treatment as fixed factor. To evaluate the effects of a disruption of the parent-offspring co-adaptation on offspring, we analyzed as response variables chick cellular lysis and agglutination and rate of increase in body mass 15 d after hatched. We fitted linear models that included as explanatory variables the swapping treatment as fixed factor and, the difference in parental provisioning rate between rearing and genetic mother and father, and chick growth rate as covariates (ulna growth from hatching to 15 d post-hatched). Some blood samples were missed in the field, handling processes, or laboratory analyses, so the sample size differ for statistical analyses on cellular lysis among family members (males n = 27, females n = 29, offspring n = 35) and agglutination (males n = 27, females n = 29, offspring n = 35).

RESULTS

Are rearing and genetic parents' provisioning correlated to offspring begging?

Provisioning by rearing parents and begging rate by reared offspring were phenotypically correlated. Both, provisioning by the rearing mother ($F_{1,36} = 10.96$, $P = 0.002$; $\beta = 0.26 \pm 0.06$, effect size = 0.30) and rearing father ($F_{1,36} = 6.33$, $P = 0.02$; $\beta = 0.14 \pm 0.04$, effect size = 0.16) increased as chick begging augmented, independently of whether the reared chick was cross-fostered or control (rearing mother: swapping treatment * begging rate $F_{1,31} = 1.08$, $P = 0.31$; rearing father: swapping treatment * begging rate $F_{1,30} = 0.44$, $P = 0.51$). Laying date and egg volume were not associated with provisioning by the rearing mother (laying date, $F_{1,34} = 0.70$, $P = 0.41$; egg volume, $F_{1,32} = 0.68$, $P = 0.42$) or the rearing father (laying date, $F_{1,31} = 1.83$, $P = 0.19$; egg volume, $F_{1,32} = 1.46$, $P = 0.24$).

Interestingly provisioning by genetic mothers, but not by genetic fathers, was correlated with the begging behavior of the genetic offspring. In the control group, genetic mother provisioning was positively associated with the begging behavior of the genetic offspring, whereas in the cross-fostered group, provisioning by the genetic mother was negatively correlated with the begging behavior of the genetic chick (Table 1a; Figure 1; effect size = 0.31). The results remained qualitatively similar when only cross-fostered nests were analyzed using as response variable the weighed rate of provisioning by the genetic mother, and as explanatory terms the weighed rate of begging by the genetic chick and the specific direction of the cross-fostering (whether chicks were swapped from a nest with a colorful male to a nest with a dull male or vice versa) (Table 1b). Provisioning by the genetic father was positively correlated with the genetic offspring begging (Table 1a). However, when only nests from the cross-fostered group were tested, genetic father weighed provisioning and genetic offspring weighed begging behavior were not longer associated (Table 1b); yet, dull fathers rearing chicks from colorful males provisioned at lower weighed rates (Table 1b). Laying date and egg volume were unrelated to the genetic mother and father provisioning or weighed provisioning rates (Table 1).

Does cross-fostering impact negatively parents and/or offspring?

The cross-fostering treatment affected the immune response of fathers, and marginally of mothers (Table 2). Mothers rearing foster chicks tended to present lower levels of cellular lysis during the period of parental care compared to mothers rearing their own genetic offspring (*P* = 0.08, effect size = 0.13, power = 0.39; Table 2a; Figure 2a). The cross-fostering treatment or the difference in begging rate between the genetic and the foster offspring did not influence agglutination response of rearing mothers (Table 2a). Fathers rearing foster offspring that begged at higher rates than their genetic chicks showed a decline in the levels of cellular lysis from courtship to the offspring rearing period (Table 2b; Figure 2b effect size = 0.24). Compared with courtship, during rearing period fathers showed lower agglutination response (mean ± S.E., courtship 2.63 ± 0.37; rearing 2.02 ± 0.26; Table 2b). The swapping treatment or the difference between reared and genetic chick begging behavior were unrelated to the rearing father agglutination response (Table 2b).

The cross-fostering treatment or the difference between genetic and rearing mother or father provisioning were not associated with offspring immune response or rate of increase in body mass (Table 3). However, chicks from genetic fathers whose provisioning was lower than the rearing father's provisioning tended to gain more body mass (*P* = 0.08; β = -0.15 ± 0.08; Table 3b). Swapping treatment or the difference between rearing and genetic mother provisioning were unrelated with chick cellular lysis or agglutination (Table 3b).

DISCUSSION

In the brown booby, rearing parents provisioning and offspring begging were positively associated. This phenotypic correlation suggests that parents respond to offspring begging by increasing provisioning rate. We previously found that chicks with lower body mass at hatching have higher begging rates to both parents resulting in higher parental provisioning, and consequently in greater offspring body mass gain (Montoya & Torres 2015). Hence, in this species begging calls might be associated with offspring condition or hunger (Grafen 1991; Johnstone 1999), as was confirmed in a sister species, the blue-footed booby (*Sula nebouxii*, Villaseñor & Drummond 2007). Further studies evaluating proximate and long term costs of begging and parental investment (Godfray & Johnstone 2000), and potential constraints of parents to increase provisioning effort, are required to understand the state of the parent-offspring conflict in this species (Royle *et al.* 2004). However, an inter-dependency of parental and offspring traits as found here, might in theory favor the selection of specific combinations of parental supply and offspring demand over others.

Interestingly, through a cross-fostering experimental design we found a negative correlation between provisioning by genetic mothers and their genetic offspring demand. Similar results were found when the weighed provisioning rate (provisioning by the genetic mother divided by the reared chick begging) and the weighed begging rate (genetic chick begging rate divided by the provisioning received by the rearing mother) were analyzed. This negative relationship might result from the fact that clutches were cross-fostered between nests where the males had contrasting gular color, a trait that is associated with paternal provisioning (Montoya & Torres 2015). However, this is unlikely because the specific direction of the cross-fostering treatment (i.e. green father to dull one or vice versa) was not significant in the analyses that tested the correlation between genetic mother provisioning and its genetic offspring begging. Furthermore, in the brown booby male gular color is unrelated to maternal provisioning and offspring begging (Montoya & Torres 2015).

Life-history traits of brown boobies, such as long-life and slow reproductive rate (delay of starting of reproduction, one offspring per annual breeding attempt), probably have favored adults that optimize resource allocation to reproduction through lifetime, imposing a stronger selective pressure on parental supply rather than on offspring demand (Kölliker *et al.* 2005). Accordingly, co-adaptation models predict that, when offspring's direct effect on parental provisioning is positive (i.e. mother's provisioning increases with offspring begging), a negative genetic correlation between parental provisioning and offspring begging is expected (i.e. combinations of good supplier mothers having low demanding genetic offspring and vice versa; Kölliker *et al.* 2005; Kölliker *et al.* 2012). In the brown booby, maternal provisioning responds to offspring solicitation, so there is opportunity for offspring to overexploit maternal generosity and selective pressure on maternal provisioning might thereby be stronger than selective pressure on offspring demand. Thus, a negative co-adaptation between mother-offspring would be the evolutionary mechanism preventing female overexploitation to occur (Kölliker *et al.* 2005; Meunier & Kölliker 2012). However, an experimental study directly manipulating begging rate is necessary to confirm the effect of begging levels on maternal provisioning and fitness.

The negative mother supply-offspring demand co-adaptation suggested by our results might have resulted through direct genetic effects or through maternal effects in the egg (i.e. an effect of the mother's phenotype or environment on offspring's phenotype through maternal investment on egg's composition; Wolf & Brodie 1998; Wolf et al. 1998). Evidence of a maternal effect favoring a mother-offspring co-adaptation was provided by Hinde *et al.* (2009), where a probable variation of maternal androgens in the egg, associated with food availability, leaded to a correlation between genetic mother provisioning and offspring begging. However, the experimental design used here does not separate these two sources of variation. Regardless of the specific mechanism, our results suggest a probable negative mother-offspring co-adaptation.

Genetic father provisioning and offspring begging were unrelated. This lack of correlation might result from maternal effects serving only to mothers' interest by enhancing mother-offspring co-adaptation (e.g. Hinde *et al.* 2009, 2010). In this scenario, mothers may provide information to the offspring regarding its own level of supply through egg composition, promoting a mother-offspring co-adaptation (Wolf & Brodie 1998; Kölliker *et al.* 2005). However, when the maternal effect is associated with signals of male provisioning capacity, or mother and father provisioning capacity are correlated such a maternal effect could also favor a father-offspring co-adaptation. In the brown booby, male and female provisioning (number of feeding events) are not correlated, but male gular color is positively associated with paternal feeding, and females lay smaller eggs when paired with colorful males (Montoya & Torres 2015). These previous findings suggest that maternal effects might favor a father-offspring co-adaptation, but no evidence for such a correlation was found in the present study. Alternatively, fathers might exert more control on offspring begging than chicks on paternal provision, as suggested by the fact that compared to mothers, fathers are more responsive (higher weighed provisioning rate) to offspring begging calls ($F_{1,38.03} = 6.93$, $P = 0.01$). Thus, if paternal proclivity to allocate food to offspring is higher than maternal one, then selective pressures might have not favored specific combinations of paternal supply-offspring demand, because offspring do not pay costs for extra-begging, or fathers for extra-provisioning. Finally, the absence of a genetic father provisioning-offspring begging correlation could be associated with the presence of extra-pair paternity, but this possibility needs to be directly assessed in the future. At present, we have no evidence of a father provisioning-offspring begging co-adaptation.

Cross-fostering treatment affected negatively immune response of fathers, and marginally of mothers. Mothers rearing foster chicks tended to have lower cellular lysis, when compared with mothers rearing their own offspring ($P = 0.08$; effect size = 0.13). Disrupting the co-adaptation between genetic mother provisioning and offspring begging might have impacted mother's immune response, because co-adaptation prevents mothers to invest in overly costly provisioning, and such investment diverts resources from self-maintenance to reproduction. Similarly, fathers rearing chicks with higher begging rates than their genetic offspring had lower cellular lysis. This result suggests that there could be a father-offspring co-adaptation promoted by a maternal effect that the present study failed to detect; or alternatively, that mother and father provisioning performance are correlated beyond the traits measured in this study, yet further research addressing this point is needed. The cross-fostering treatment probably induced a phenotypic mismatch between parents and offspring affecting negatively parental immune response. Thereby, provisioning above the optimal level might threaten parental fitness prospective through an impaired immune response. On the other hand, offspring were not negatively affected by the cross-fostering treatment. There was a tendency for offspring to gain more body mass when reared by fathers that provisioned more than the genetic fathers, suggesting offspring may gain short-term benefits when reared by good suppliers. Thus, in the brown booby the presumably mother-offspring co-adaptation may serve mainly the interests of parents by reducing potential extra-costs of parental care.

In summary, we found a positive phenotypic correlation between parental supply and offspring demand, and a negative correlation between genetic mother provisioning and offspring begging. Disrupting the correlation between genetic mother-offspring had a negative impact on immune response of fathers and probably of mothers, but not of offspring. To understand the family dynamics over provisioning, further studies are required exploring the mechanisms behind the mother-offspring correlation (i.e. direct genetic or maternal effects), and the fitness consequences for all the family members. Overall, the correlation between provisioning by genetic mothers and genetic offspring's begging behavior suggests that these traits could not evolve independently probably influencing the evolutionary trajectories of parental care in the brown booby.

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Table 1. Parental provisioning and genetic offspring begging.

	Female		**Male**	
a. Provisioning (all nests)	***F***	***P***	***F***	***P***
Swapping treatment	**16.67**	**<0.001**	0.23	0.64
Egg volume	0.45	0.51	0.001	0.97
Laying date	0.11	0.75	1.37	0.25
Begging rate	**7.93**	**0.008**	**4.00**	**0.054**
Swapping treatment * Begging rate	**14.82**	**<0.001**	1.00	0.32
Error df final model	31		32	

	Female		**Male**	
b. Weighed provisioning (cross-fostered nests)	***F***	***P***	***F***	***P***
Swapping direction*	0.46	0.51	**5.75**	**0.03**
Egg volume	0.42	0.53	0.26	0.62
Laying date	1.20	0.29	2.72	0.12
Weighed begging rate	**4.93**	**0.04**	2.41	0.14
Swapping direction(1) * weighed begging rate	1.02	0.33	0.47	0.50
Error df final model	16		17	

* From a nest with a colorful father to a nest with a dull male or vice versa.

Link between genetic parents provisioning and genetic chick begging was tested using generalized linear models (panel a). Weighed provisioning rate by genetic parents and weighed begging by genetic offspring was analyzed using general linear models (panel b). Clutches were either cross or mock-fostered (swapping treatment) to distinguish genetic from environmental effects. Table shows *F* and *P* values from variables in the initial model at the moment of their exclusion and in bold, variables in the final model.

Table 2. Effect of the cross-fostering treatment on immune response of parents.

	Lysis		Agglutination	
a. Rearing mother	***F***	***P***	***F***	***P***
Time	0.46	0.50	0.07	0.80
Time * Swapping treatment	**3.27**	**0.08**	2.49	0.13
Time * Begging difference	0.74	0.40	1.56	0.23
Error df final model	23		23	

	Lysis		Agglutination	
b. Rearing father	***F***	***P***	***F***	***P***
Time	**11.12**	**0.004**	**7.06**	**0.02**
Time * Swapping treatment	1.19	0.29	0.11	0.75
Time * Begging difference	**5.17**	**0.04**	0.08	0.79
Error df final model	16		19	

Repeated measures models were used to examine the impact of the cross-fostering treatment on immune response change from courtship to parental care (15 days after offspring hatched) of the rearing mother (panel a), and the rearing father (panel b). Begging difference was estimated as the begging rate of the genetic chick minus the begging rate of the genetic one. Table shows *F* and *P* values from the within-subjects effects in the initial model at the moment of their exclusion and in bold, variables in the final model.

Table 3. Effect of the cross-fostering treatment on offspring immune response and mass gain (g/day).

	Lysis		Agglutination		Mass gain rate	
	F	***P***	***F***	***P***	***F***	***P***
Swapping treatment	0.58	0.45	0.13	0.72	0.63	0.43
Genetic and rearing mother difference in provisioning	0.53	0.47	0.18	0.67	0.44	0.51
Genetic and rearing father difference in provisioning	0.92	0.35	1.04	0.32	**3.35**	**0.08**
Ulna growth rate	0.37	0.55	1.12	0.30	**24.99**	**<0.001**
Error df final model	34		34		34	

Linear models examining the effect of the cross-fostering treatment on the offspring immune response and rate of increase in body mass 15 days after hatched. Provisioning difference was estimated as the provisioning of the genetic mother or father minus provisioning of the rearing mother or father. Table shows *F* and *P* values from variables at the moment of their exclusion and in bold, variables in the final model.

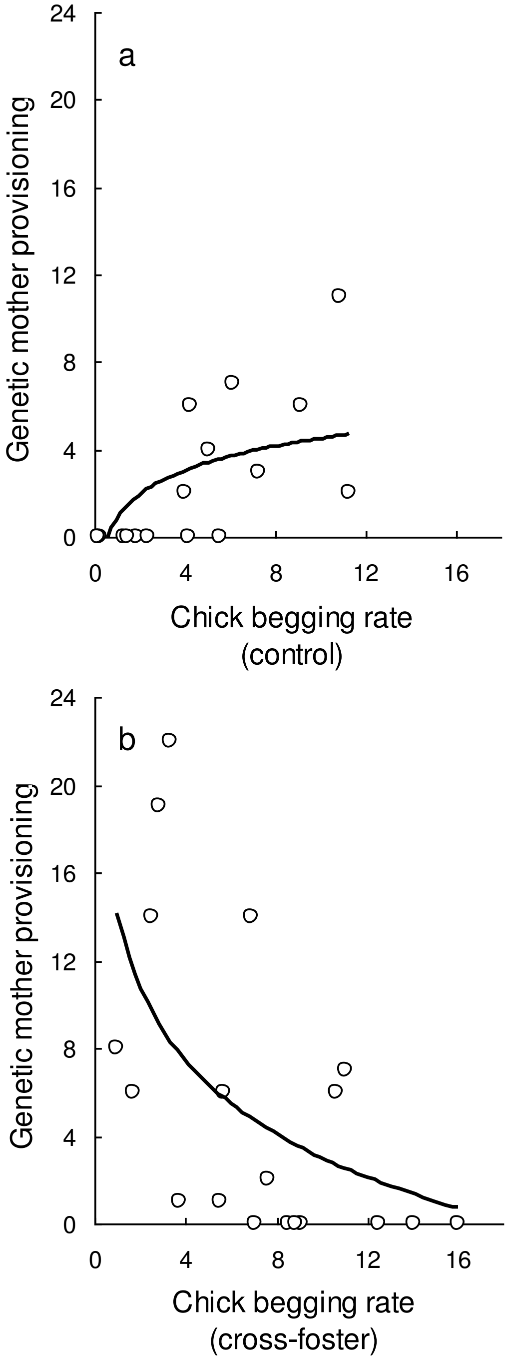

Figure 1. Relationship between mother provisioning and chick begging. (a) Genetic mother provisioning and begging of the genetic offspring in control nests (i.e. offspring remained in its original nest). (b) Provisioning by the genetic mother and begging of the genetic offspring in cross-fostered nests. Results from a negative binomial GLM with link log function (swapping x begging, $F_{1,31}$ = 14.82, $P < 0.001$, effect size = 0.31).

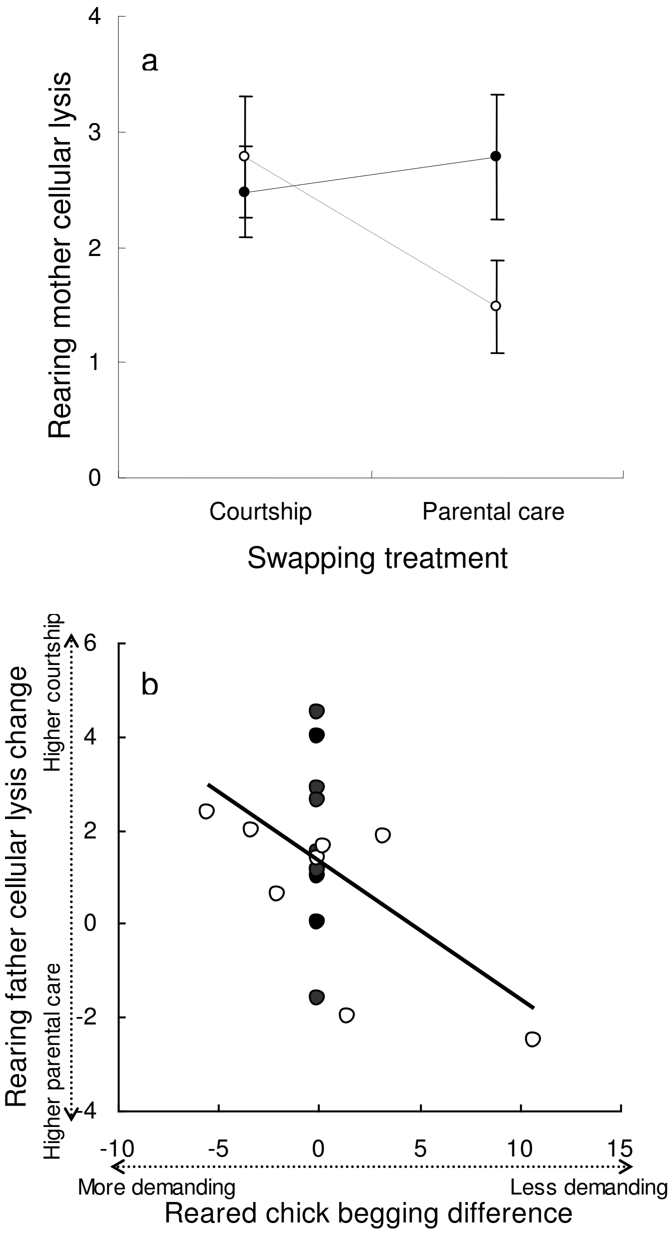

Figure 2. Effect of cross-fostering treatment on immune response in (a) mother and (b) father. As an indicator of immune response the percentage of cellular lysis inflicted to an external agent was measured at courtship and parental care. Open dots correspond to cross-fostered nests and closed ones to controls. Negative values of cellular lysis indicate lower response at courtship than at parental care (and vice versa for positive values). Negative values of reared chick begging indicate higher begging of the reared chick compared to the

genetic one (and vice versa for positive values). In panel b, the point data at the extreme right of the plot might be an outlier ($P = 0.05$); however, dropping this data point did not change the result ($F_{1,15} = 6.10$, $P = 0.03$). Effect size = 0.13 and 0.24 for panels a anb respectively.

CAPÍTULO VI

Discusión General y Conclusiones

DISCUSIÓN GENERAL Y CONCLUSIONES

Durante la comunicación los animales usan señales para proporcionar y recibir información que no se encuentra disponible directamente (Maynard-Smith & Harper 2003). Algunas de estas señales tienen el potencial para variar en periodos breves de tiempo ajustandose a cambios ocurridos en la información que proporcionan (Bro-Jørgensen 2009). Por ejemplo, duante la elección de pareja, los individuos usan señales para evaluar la magnitud de los beneficios que una pareja potencial ofrece, algunas de estas señales varian en el transcurso de horas reflejando cambios en la condición del individuo (Rosen & Tarvin 2006; Velando *et al*. 2006). Durante los cuidados parentales, los padres usan señales proporcionadas por la cría, como las llamadas de solicitud, para ajustar la provisión de alimento en función de la condición o necesidad de los hijos (Grafen 1991; Johnstone 1999). En esta tesis se evaluó, por una parte, el potencial del color gular del bobo café para informar sobre el estatus oxidante del individuo y sobre la magnitud de los beneficios ofrecidos a las parejas reproductivas potenciales. Por otra parte, se exploró la oportunidad para la ocurrencia de una coadaptación entre las señales de solicitud de las crías y la disposición de los padres a proveer alimento.

Compromisos fisiológicos

Como alternativa a la existencia de un compromiso de recursos entre el mantenimiento somático y la inversión reproductiva, el estrés oxidante fue propuesto como mecanismo subyacente al costo de la reproducción, motivando en los últimos 15 años un crecimiento en el número de estudios experimentales

(Revisado en Metcalfe y Monaghan 2013) y debates conceptuales sobre el tema (Speakman y Selman 2011; Speakman y Garratt 2014; Blount *et al.* 2015).

Usando una aproximación longitudinal, en el capítulo II se encontró que machos y hembras de bobo café presentaron una menor producción de radicales libres durante la crianza comparados con sus propios niveles durante el cortejo, mientras que, el daño oxidante en lípidos en los dos sexos, antes y después del esfuerzo parental no difirió. Estos resultados sugieren que no hay un costo oxidante asociado a la inversión en cuidados parentales, pero podrían indicar la existencia de un mecanismo de protección contra el daño oxidante durante la reproducción (Blount *et al.* 2015). Durante la crianza, la producción de radicales libres podría disminuir como resultado de la acción efectiva de la respuesta antioxidante hasta el agotamiento, en consecuencia, esta respuesta podría registrar un declive como ha sido reportado (Alonso-Alvarez *et al.* 2004). Otra vía posible, es que una menor producción de radicales libres sea interpretada por el organismo como una reducción en la amenaza oxidante, generando una regulación a la baja en la reserva de antioxidantes como ha sido encontrado previamente (Wiersma *et al.* 2004). Alternativamente, la capacidad antioxidante realmente podría mejorar durante la reproducción (Bize *et al.* 2008; Beaulieu *et al.* 2011; Garratt *et al.* 2013), neutralizando el exceso de radicales libres de forma efectiva desde etapas tempranas de su producción. La existencia de un mecanismo protector contra el daño oxidante permitiría explicar porqué el nivel de daño en lípidos no difirió significativamente antes y durante el esfuerzo parental (Capítulo II), e incluso porqué en otros estudios el daño oxidante en algunos substratos parece dismunir durante la reproducción (Nussey *et al.* 2009; Oldakowski *et al.* 2012; Aloise-King *et al.* 2013; Garratt *et al.* 2013; Costantini *et al.* 2014). En resumen, no se encontró evidencia de que la incubación y la crianza impongan costos oxidantes, futuros estudios podrían evaluar el papel de los mecanismos de protección contra el estrés oxidante en este resultado.

Por otra parte, usando una aproximación transversal se encontró que las madres de bobo café con mayores niveles de ROS en cortejo pusieron huevos más pequeños y criaron pollos con menor ganancia de masa corporal, además, madres con mayor daño en lípidos en cortejo cuidaron menos tiempo de las crías (Capítulo II). Los niveles de estrés oxidante al inicio del evento reproductivo podrían limitar la inversión reproductiva posterior de las madres, probablemente protegiendolas de sufrir una mayor amenaza oxidante, pero comprometiendo su inversión en la cría. Estos resultados sugieren que el estrés oxidante podría mediar los compromisos entre carcterísticas de historia de vida en las hembras.

De forma alternativa a la existencia de un mecanismo protector del daño oxidante, la reducción en el nivel de radicales libres registrados durante la crianza podría ocurrir si en el bobo café, la inversión reproductiva previa a la eclosión fuera más costosa que la inversión posterior. La larga expectativa de vida y baja tasa reproductiva (inicio tardío de la reproducción, una cría producida por intento reproductivo anual) de esta especie, podrían imponer costos superiores por la inversión reproductiva temprana (búsqueda y elección de una pareja reproductiva, ubicación y defensa de un lugar de anidación, establecimiento de una puesta temprana) en comparación con los que impone el esfuerzo parental, porque las decisiones reproductivas tempranas pueden tener un papel decisivo en la culminación exitosa del evento reproductivo. Esta interpretación coincide con la correlación positiva encontrada en los dos sexos, entre inversión en color tegumentario en cortejo y daño en lípidos (Capítulo II), así como con el hecho de que machos y hembras presentaron mayores niveles de radicales libres cuando se encontraban más próximos a la fecha de puesta y cuando establecieron puestas más tempranas en la temporada (Capítulo II). Finalmente, es necesario considerar la posibilidad de que un reduccion en la producción de radicales libres podría tener un efecto perjudicial, debido al papel de estos en la señalización celular. Nuevos estudios podrián explorar esta idea.

Durante el cortejo machos de bobo café con gulares más verdes y hembras con gulares más brillantes presentaron mayor daño en lípidos (Capítulo II). En esta especie, la inversión en colores tegumentarios podría generar costos oxidantes. Or consiguiente, solo individuos en buena condición podrían soportar, de manera temporal, un incremento en el daño en lípidos como costo próximo de la inversión en color, como se esperaría a partir de la hipótesis del *handicap* (Zahavi 1975; Grafen 1990). Los individuos más ornamentados podrían tener mecanismos de reparararación del daño oxidante más eficientes o mejores estrategias de forajeo (Michael *et al.* datos sin publicar) y por este motivo, podrían pagar el costo oxidante por exhibir ornamentos más vistosos. En este contexto, es posible que los individuos más onamentados además fueran más longevos, como se ha encontrado en otras especies (Hõrak *et al.* 2001; Pike *et al.* 2007). De esta forma, se esperaría que las hembras prefirieran aparearse con machos más coloridos, debido a que estos machos podrían ofrecer mayores beneficios (ver discusión sección *"función"*). Por otra parte, las hembras de bobo café podrían pagar el costo oxidante (i.e. daño en lípidos) por exhibir gulares más brillantes para atraer a machos más coloridos, considerando la ocurrencia de apareamiento concordante por color en la colonia estudiada (Montoya, B. y Torres, R. datos sin publicar) y la oportunidad para la elección de pareja mutua en esta especie (Burley 1977; Amundsen 2000; Kokko y Johnstone 2002). En consecuencia, las ganancias en términos de éxito reproductivo por invertir en tegumentos coloridos podrían exceder sus potenciales costos oxidantes en los dos sexos.

La inversión en color, ligada a la asignación de carotenoides, resultó estar restringida al cortejo en los dos sexos, ya que durante la eclosión y el cuidado parental los individuos presentaron gulares menos verdes y más azules (Capítulo IV, Anexo I). Durante el cortejo la asignación de pigmentos carotenoides al tejido gular podría ser mayor que durante la crianza, promoviendo un incremento de la reflectancia en verde y de la absorbancia en azul, debido a la estructura química de estas moléculas (Fox 1979; McGraw 2006). En un estudio reciente, el verde del gular de los machos se relacionó con nichos de forrajeo más distantes y de mayor nivel trófico y, en las hembras la explotación este mismo tipo de nichos se relacionó con el verde el gular de su pareja (Michael *et al.* datos sin publicar). Por lo tanto, individuos con mejores coloraciones prodrían ser más capaces de enfrentar los altos costos de forrajeo de realizar viajes a nichos más alejados o alternativamente, podrían ser forrajeadores más eficientes como se ha observado en otras especies (e.g. Karino *et al.* 2005).

Función

En el capítulo III, por medio de un experimiento de intercambio de puestas se encontró que machos con gulares más verdes cuidaron más tiempo de las crías y las alimentaron más, adicionalmente, tuvieron hijos adoptivos que ganaron más peso e hijos genéticos que alcanzaron mayor tamaño estructural (Capítulo II). Estos resultados sugieren que al evaluar el color del gular de los machos, las hembras de bobo café podrían obtener información acerca de la magnitud de los beneficios directos e indirectos que un individuo puede proporcionar. De forma consistente con esta idea, cuando se utilizó un modelo de percepción visual en aves para estimar el color del gular, los machos que lograron establecer una puesta tuvieron gulares que produjeron mayor recepción de fotones (Capítulo IV), sugiriendo que la inversión en color podría asociarse con variables que favorecen el establecimiento de una puesta. Adicionalmente, los machos más ornamentados, al tener hijos que ganan más tamaño estructural y peso, podrían tener mayor éxito en el evento reproductivo actual, ya que las condiciones experimentadas en etapas tempranas del desarrollo han mostrado tener efectos a largo plazo sobre la adecuación de los individuos en diferentes especies (Gustafsson *et al.* 1995; de Kogel *et al.* 1997; Metcalfe y Monaghan 2001; van de Pol *et al*. 2006; Reid *et al.* 2010; Boonekamp *et al.* 2014).

El potencial de esta señal dependiente de carotenoides para informar sobre beneficios directos, podría estar relacionado con la presencia de variación ambiental asociada a su producción como lo sugiere su relación con la fecha de captura (Capítulo IV) y el nicho de forrajeo (Michael *et al.* datos sin publicar). Adicionalmente, variabilidad genética asociada por una parte, a la capacidad para obtener fuentes de alimento ricas en carotenoides, extraer, absorber y movilizar estas moléculas (Olson y Owens 1998; Karino *et al.* 2005; Pérez-Rodríguez 2008), y por otra parte, a la capacidad para reparar daño oxidante sin comprometer la viabilidad, podría relacionarse con los beneficios indirectos señalados por la coloración gular. Hasta qué punto los potenciales beneficios indirectos señalados por la coloración del gular son favorecidos por un efecto materno es un tema de futuros estudios.

Coadaptación

Durante los cuidados parentales, la tasa de provisión de los padres impone una presión de selección sobre la variación en la conducta de solicitud de las crías, de la misma forma, las solicitudes de las crías ejercen una presión de selección sobre la tasa de alimentación de los padres. Debido a esta interdependencia, la conducta de los padres y de la cría tienen efectos epistáticos sobre la adecuación de las dos partes, favoreciendo el éxito de determinadas combinaciones provisión-solicitud sobre otras combinaciones o sobre variaciones independientes en cada componente (Wolf y Brodie 1998; Kölliker *et al.* 2005).

En el Capítulo V, usando un diseño de intercambio de puestas, se encontró que la tasa de solicitud de las crías se correlacionó positivamente con la tasa de provisión de los dos, padre y madre cuidadores, tanto en los nidos controles como en los intercambiados. Estos resultados sugieren que, en el bobo café, la tasa de entrega de alimento es sensible a las señales de solicitud de las crías. La solicitud de alimento en las crías en esta especie podría ser una señal de necesidad, a juzgar por la relación negativa encontrada entre peso corporal a la eclosión y la tasa de solicitudes a los dos padres (Capítulo II). De forma interesante, en los nidos intercambiados, la tasa de alimentaciones de la madre genética (a una cría adoptiva) se relacionó negativamente con la tasa de solicitudes de su cría genética (a una madre adoptiva, Capítulo V), sugiriendo la existencia de una coadaptación negativa entre madre y cría (Agrawal *et al.* 2001; Kölliker *et al.* 2005).

Los modelos de coadaptación entre la provisión de la madre y la solicitud de la cría, predicen que cuando existe una presión de selección más fuerte sobre la provisión de los padres que sobre la solicitud de las crías, los padres incrementan la provisión de alimento cuando las crías solicitan a tasas más altas, y como mecanismo evolutivo que previene la sobre-explotación parental, las combinaciones de padres genéticos buenos provisionando con crías que solicitan a tasas bajas (y viceversa) son más exitosas en promedio que otras combinaciones padres-cría (Kölliker *et al.* 2005; Kölliker *et al.* 2012). Las combinaciones de madres buenas proveedoras con crías poco solicitadoras, así como de madres malas proveedoras con crías demandantes podrían haber resultado más favorecidas que otras combinaciones, al ahorrar costos extra por una alta tasa de solicitud en el primer caso, y asegurar la provisión materna en el segundo, afectando positivamente la adecuación de las dos partes cría (Phillips & Arnold 1989; Kölliker *et al.* 2005; Kölliker *et al.* 2012). En apoyo a esta interpretación, la disrrupción de la correlación entre alimentaciones de la madre genética y solicitudes de la cría afectó negativamente la respuesta inmune del padre y marginalmente la de la madre (Capítulo V). Es posible que en el bobo café, la ocurrencia de un solo evento reproductivo anual y el número reducido de crías sobrevivientes por cada evento, favorezcan una mayor presión de selección por parte de la cría sobre las alimentaciones de los padres, como se ha observado en otras especies con cacterísticas de historia de vida similares (Maestripieri 2004).

Contrario a lo esperado en ausencia de una correlación entre las alimentaciones del padre genético y las solictudes de la cría, el intercambio de puestas afectó negativamente la respuesta inmune del padre (Capítulo V). Este resultado, podría responder a que la disrrupción de la coadaptación madre-cría afectó de manera colateral la dinámica de alimentaciones por parte de los machos. Considerando que las solitudes de la cría a los dos padres estan relacionadas positivamente y que en los dos padres, la tasa de provisión está asociada positivamente con la tasa de solicitud de las crías (Capítulo III), un cambio en la dinámica de la interacción madre-cría podría tener efectos sobre la interacción padre-cría, por medio de la tasa de solicitud del pollo. Alternativamente, un efecto materno podría promover tanto una coadaptación madre-cría, como padre-cría, en caso de que la hembra modificara la asignación de recursos al huevo en respuesta a los ornamentos masculinos, y que esta asignación, tuviera efecto sobre la tasa de solicitud de las crías. De acuerdo con esta idea, las hembras emparejadas con machos con gulares más verdes, ornamento que se relaciona con la capacidad del macho para provisionar alimento, pusieron huevos más pequeños y las crías de huevos mas pequeños solicitaron más a los padres que las crías de huevos más grandes (Capítulo III). En este esenario, es probable que el presente estudio no haya logrado identificar la ocurrencia de una coadaptación padre-cría debido a falta de poder estadístico, ausencia de información sobre la característica de la provisión paterna relevante o presencia de paternidades extra-pareja (Capítulo

V). En otras especies se ha observado una interacción entre antagonismo parental (i.e. ventaja relativa de un alelo sobre otros dependiendo del sexo del padre del que es heredado) y coadaptación madre-cría sobre la asignación de cuidados parentales (p. ej. Meunier y Kölliker 2012); sin embargo, la ocurrencia de esta interaccion no pudo ser explorada en el presente estudio. Para comprender mejor la dinámica de la interacción familiar durante el cuidado parental en el bobo café, es necesario un estudio en el que se evalúe directamente el mecanismo detrás del proceso de coadaptación madre-cría (p. ej. efectos maternos o genéticos), se investigue en más detalle la potencial coadaptación padre-cría y se determine la ocurrencia de costos en adecuación para los padres y/o la cría como resultado de la ruptura del proceso de coadaptación madre-cría.

Conclusiones

En el bobo café, podrían existir compromisos en la inversión entre diferentes etapas del evento reproductivo mediados por el estrés oxidante, ya que:

- Machos y hembras presentan niveles de ROS más altos durante el cortejo que durante la crianza; mientras que en los dos sexos, el nivel de daño en lípidos no difiere significativamente entre estas dos etapas reproductivas.

- Machos y hembras que establecen puestas tempranas en la temporada reproductiva y que se encuentran próximos a la fecha de puesta presentan mayores niveles de producción de ROS.

- Durante el cortejo, machos con gulares más verdes y hembras con gulares más brillantes presentan mayores niveles de daño oxidante en lípidos.

- Hembras con mayores niveles de ROS y daño oxidante en lípidos, ponen huevos más pequeños y permanecen menos tiempo atendiendo a la cría respectivamente.

El color del gular en machos y hembras de bobo café parece variar a lo largo del evento reproductivo, posiblemente porque esta característica tiene mayor valor informativo durante el cortejo que en etapas posteriores, ya que:

- Machos y hembras presentan gulares con mayor reflectancia en verde y menor en azul durante el cortejo; mientras que, al momento de la eclosión y durante la crianza el gular en los dos sexos exhibe menor reflectancia en verde y mayor en azul.
- Machos con gulares más verdes cuidan más tiempo de sus crías y las alimentan más; además, sus crías ganan más peso corporal y tamaño estructural.

Las interacciones entre madres y crías de bobo café podrían estar moduladas por la ocurrencia de una coadaptación entre las señales de solicitud emitidas por la cría y la disposición de las madres para proveer alimento , ya que:

- Durante la crianza, machos y hembras responden positivamente a las solicitudes de las crías en el nido. Sin embargo, al intercambiar las nidadas, la tendencia de las madres genéticas a alimentar se relaciona negativamente con la tendecia de sus críos genéticos a solicitar.
- Al romper la aparente coadpatación entre la madre y la cría, se observó un adisminución en la respuesta inmune de los machos, y marginalmente de las hembras.

En resumen, esta tesis sugiere la existencia de compromisos fisiologicos mediados por el estrés oxidante entre diferentes etapas de la inversión reproductiva, el uso de ornamentos coloridos como señal de beneficios directos e indirectos para las hembras y la ocurrencia de una coadaptación entre la provisión materna y la solicitud de las crías en el bobo café.

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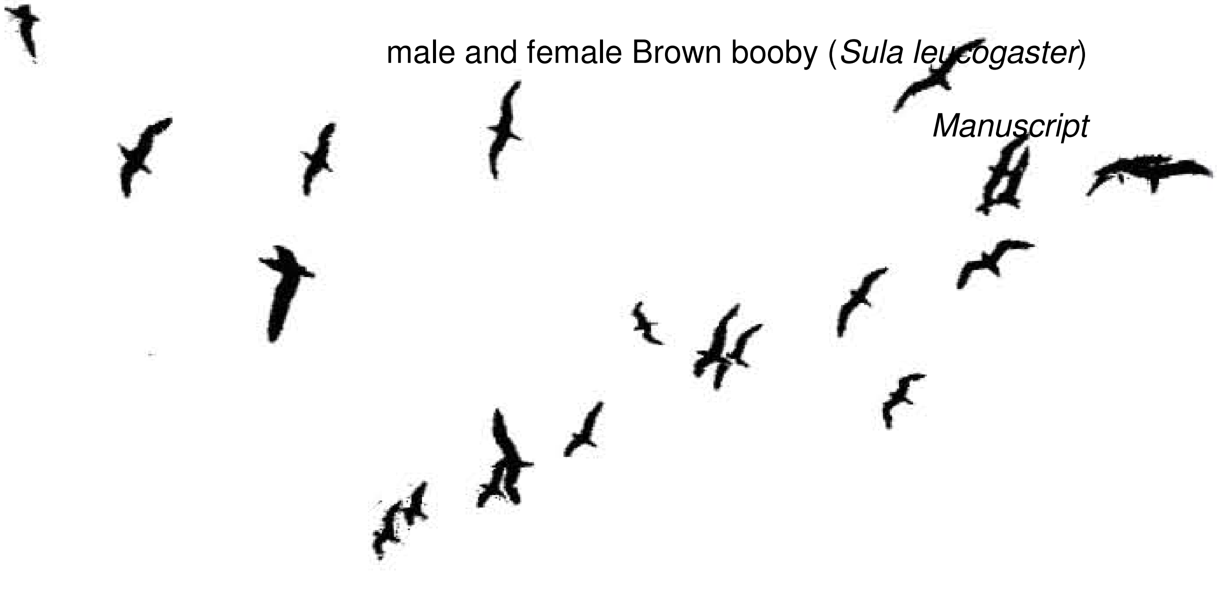
ANEXO I

HPLC determination of carotenoid pigments in gular skin of male and female Brown booby (*Sula leucogaster*)

Manuscript



HPLC determination of carotenoid pigments in gular skin of male and female Brown booby (*Sula leucogaster*)

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ABSTRACT

Carotenoid-dependent colors in teguments have received great attention because of their potential for dynamically mirror changes in individual's condition. We used HPLC to determine the presence of carotenoids in the gular tissue of four brown booby (*Sula leucogaster*) males and two females during courtship. We identified four carotenoid compounds: lutein, zeaxanthin, 13 cis-beta-carotene and trans-gamma-carotene in the gular tissue of both sexes, being the most abundant 13 cis-beta-carotene. An interaction between caroteoind deposition and structural color may be the responsible for the green-bluish coloration exhibited for males of this species during courtship in the gular skin. The identification of carotenoid deposition in male gular skin opens new questions about the evolution and function of this color as a signal in the context of sexual selection.

Key words: Integument, condition dependent, carotenoids, color, signal.

Carotenoid-dependent colors have been in the focus of behavioral biologists' attention since they were proposed as good examples of condition-dependent ornaments. These molecules cannot be synthesized *de novo* by vertebrates, being principally available for good foragers (Hill 1990; Olson & Owens 1998; Casagrande *et al.* 2006). Once acquired through diet, the allocation of carotenoids to ornaments depends on, the efficiency of the individual to absorb and mobilize them, the necessity to allocate them to other functions as the immune or antioxidant responses, and the capacity to deal with their toxic potential (Lozano 1994; Olson & Owens 1998; Møller *et al.* 2000; Simons *et al.* 2014). Therefore, changes in one or more of these processes might affect the quality of carotenoid-dependent colors. Color in feathers frequently appears months before mate choice occurs and stays relatively unmodified despite the fact that the individual's condition can improve or deteriorate after molting. In contrast, coloration in fleshy structures has shown the potential to be dynamically updated as a function of seasonal changes (Negro *et al.* 1998; Pérez-Rodríguez 2008), variations in nutritional condition (Velando *et al.* 2006; Costantini *et al.* 2007; Pike *et al*, 2007), immune response (Faivre *et al.* 2003; McGraw & Ardia 2003; Baeta *et al.* 2008) and oxidative balance (Torres & Velando 2007; Alonso-Alvarez & Galván 2011). These characteristics have motivated the use of carotenoid-dependent colors iin fleshy structures as frequent examples of condition dependent characters (Lozano, 1994; von Schantz *et al.* 1999; Hõrak *et al.* 2001 Badyaev & Hill 2000; McGraw *et al.* 2003).

The brown booby *Sula leucogaster* is a long-lived seabird distributed throughout the tropics. Adults exhibit sexual dichromatism in their plumage

(Nelson 1978), and the color of the bare skin (i.e., lores, cheek, gular and feet). During courtship, female gular skin is green-yellowish and male's is green-bluish (Montoya and cols. unpublished data). In one of the most frequent courtship displays, the male's gular skin coloration is exhibited to potential mates by beak elevations pointing to the sky accompanied by acute vocalizations. Brown boobies are a socially monogamous species with a long period of obligated bi-parental care (Nelson 1978; Osorno & Drummond 2003; Tershy & Croll 2000). Males and females participate in clutch incubation, and offspring attendance and feeding during a period of up to five months (Nelson 1978).

Since male gular skin color can potentially play a role as signal of benefits for females, in this study, we aimed to identify whether there is deposition of carotenoid pigments in the gular skin of brown booby males and females during courtship. Traditionally, red, orange and yellow colors have been thought to rely strongly on pigments such as carotenoids, while blue colors have been proposed to depend on structural arrangements (Brush 1990; Bagnara *et al*. 2007). Hence, we expected to find carotenoid deposition in female gular skin. However, skin color depends on the interaction between structural reflectance and pigmentary mechanisms (Prum & Torres 2003), and some blue-greenish skin colors in birds have been found to result from the interaction between short wavelength structural reflectance and pigments deposited in more superficial layers, that is the case in the phylogenetically closely related species, the blue footed booby (*Sula leucogaster*, Velando *et al*. 2006). Hence, despite the green-bluish appearance of the brown booby male skin, we expect to find carotenoid deposition as likely responsible for the greenish coloration when interacting with the blue structural color in males.

METHODS

Tissue samples were collected in the Brown Booby breeding colony at Isla Larga, Parque Nacional Islas Marietas Nayarit, México (20º 41'N 105º 36' W), during the reproductive season of June, 2010. Gular skin samples of four males and two females were obtained less than 10 hours after the natural death of the individual. Samples were individually kept in liquid nitrogen for about three months and then stored at -70ºC until HPCL analyses were performed.

Sample preparation

Prior to HPLC analysis, 0.5 gr of each tissue sample was separated and individually liquefied in a porcelain mortar while adding liquid nitrogen and 3 mL HEAT Hexane: Ethanol: Acetone: Toluene (10:6:7:7 v/v). Product of the maceration was transferred into individual tubes and sonicated continuously for 5 mins. Then, samples were saponified by adding 0.5 mL of KOH (40% v/v) and incubated at 56 °C in a water bath for 20 min. Samples were removed from the water bath and 3 mL hexane was added. Samples were then vortexed for 30 seconds and the volume was adjusted to 10 mL using Na_2SO_4 (10% v/v). The superficial phase of the solution was transferred into a new tube and evaporated using pressurized air. Finally, samples were solubilized in 50 µL hexane.

High Performance Liquid Chromatography (HPLC)

Previously prepared samples were injected via a 20 µL loop using a 100 µL syringe in an HPLC System (Hewlett-Packard HPLC System 1100A Series, Wilmington, DE, USA) fitted with an ODS Allsphere column (1.5 µL, 4.6 mm × 150 mm; Alltech PN 778357, Nicholasville, NY, USA). Data were recorded from 200 – 600 nm using a photodiode array detector (DAD, HP 3D Chemstation program). We used an isocratic elusion system (Hewlett-Packard HPLC quanton pump system 1100A Series, Wilmington, DE, USA) at a constant flow rate of 1.2 mL min^{-1} for 15 min. The mobile phase A was acetonitrile: methanol: TRIS HCl 0.1 M pH 8 (72:8:3 v/v) and mobile phase B was methanol: hexane (40:10 v/v).

Pigment identification and quantification

Principal pigment identification was performed for the main four HPLC peaks by comparing their respective retention times with authentic lutein, zeaxanthin, β- and α-carotene external standards (Sigma-Aldrich, St. Louis, MO, USA). Peaks that differed from the external standards injected were identified by comparing their relative retention time and maximum absorbance spectra with previous reports for derived forms of carotene (Inbaraj *et al*. 2008, Rojas-Garbanzo *et al*. 2011, Hsu *et al*. 2012). Estimation of carotenoid content in gular skin (µg/g) was based on the formula (Stradi *et al*. 2001): (A * extract volume (mL) x 10^4)/(E * tissue mass (gr)), where A is the absorbance at wavelength max of the sample and E is the extinction coefficient 1%/1cm at λ max of the relevant carotenoid (2500 for unidentified caretenoids, Britton 1985).

RESULTS

The presence of four types of carotenoid compounds was identified in male gular skin samples: Lutein (rt = 5.79, λ max = 448-474), zeaxanthin (rt = 6.54, λ max = 454-480), 13 cis-beta-carotene (rt = 9.67, λ max = 448-474) and trans-gamma-carotene (rt = 12.44, λ max = 448-472)(Figs. 1 and 2). The identity of the pigments was confirmed by comparing them with previous reports of their retention time and maximum absorbance peaks (Inbaraj *et al.* 2008; Rojas-Garbanzo *et al.* 2011; Hsu *et al.* 2012). On average, total amount of carotenoids in male gular skin was 24.28 µg/g. From this quantity, 64.04% corresponded to cis-beta-carotene, 21.01% to trans-gamma-carotene, 9.66% to zeaxanthin and 5.24% to lutein.

In female gular skin samples carotenoids were also detected, but the total concentration was not high enough to reliably determine the identity of the main peaks. Total concentration of carotenoids in female gular skin was on average 0.58 µg/g, and the main peaks detected were 1 (rt = 6.13, λ max = 454-480), 2 (rt = 7.73, λ max = 448-470) and 3 (rt = 9.31, λ max = 448-474).

DISCUSSION

In the brown booby, male gular skin color during courtship appears to be carotenoid-dependent, providing support for the idea that blue-greenish colors can result from the interaction between structural blue color and carotenoid pigments. Opposite to what would be expected by the yellowish appearance of the female gular skin, the carotenoid concentration in female skin was lower than in male skin, suggesting that yellow colors might sometimes be structural rather than pigmentary. As carotenoid pigments in vertebrates are obtained through the diet, probably in the brown booby, greener males have better foraging abilities as suggested a recent stable isotope analysis (Michael *et al*. unpublished results), and has been shown in other species (Karino *et al*. 2005; Casagrande *et al*. 2006). Inheritance of foraging abilities for carotenoid-rich resources has been demonstrated in fish suggesting that besides direct benefits, such as better nutritional condition for the offspring, carotenoid-dependent colors might signal indirect benefits as well (Karino *et al*. 2005; Karino *et al*. 2007). Therefore, in the brown booby male gular skin may be a signal for direct and indirect benefits for females. In male brown boobies, color resulting from the interaction between structural arrangement and carotenoid deposition might be dynamically updated in response to variation in individual condition, and allow reproductive mates to adjust their investment in response to such changes. Identification of these pigments in male gular skin opens several pathways to explore regarding the function and evolution of male and female skin color in the brown booby.

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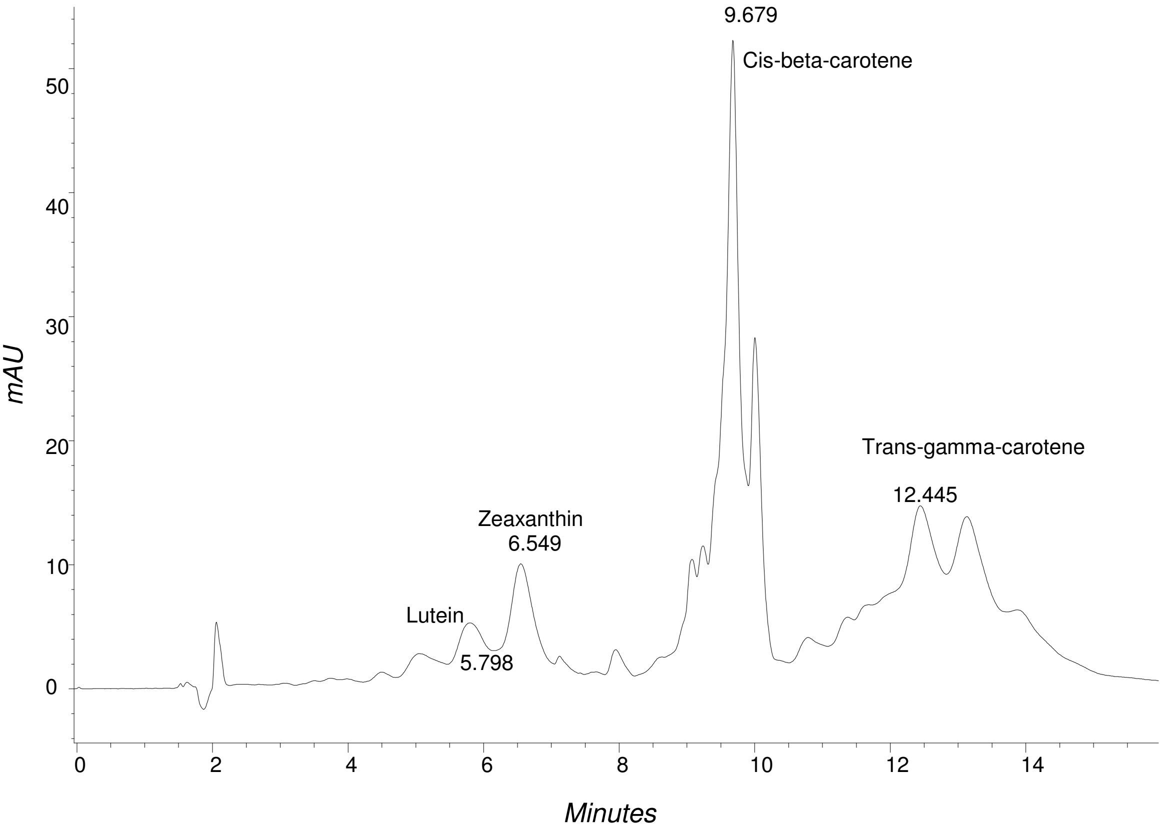

Figure 1. Representative chromatogram of the carotenoid pigments present in the gular skin of brown booby males during courtship. Carotenes were more abundant than xanthophylls.

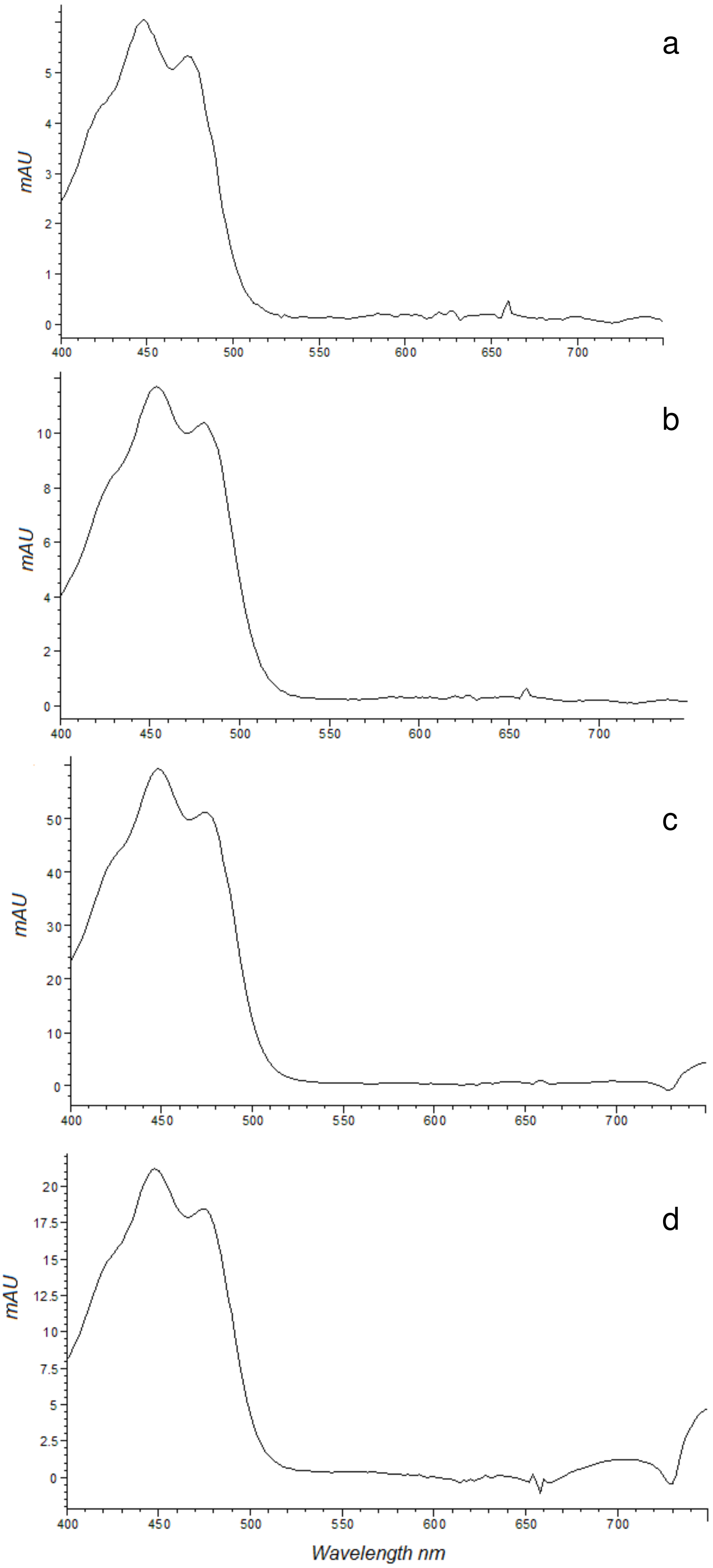

Figure 2. Main carotenoids detected in the gular skin of brown booby males during courtship. (a) Lutein, (b) zeaxanthin, (c) 13 cis-beta-carotene and (d) trans-gamma-carotene.

ANEXO II

Baseline glucose increases due to adverse developmental and adult environments and shortens lifespan in zebra finches

Submitted to Fuctional Ecology

Baseline glucose increases due to adverse developmental and adult environments and shortens lifespan in zebra finches

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Running title: Baseline glucose: Life conditions and lifespan

Summary

1. Glucose is the main energy source for cells and plasma glucose level is therefore tightly regulated. Epidemiological research in humans highlights the association of high baseline glucose levels with numerous pathologies and lifespan. Moreover, restricted access to nutrients during development has been proposed to play a key role in glucose metabolism in adulthood. Therefore, causes and consequences of individual variation in baseline glucose level are of interest as factor connecting developmental conditions to lifespan and health.
2. We tested to what extent baseline plasma glucose level is a repeatable trait in adult zebra finches, and whether glucose level was associated with age, manipulated environmental conditions during development (rearing brood size) and adulthood (foraging effort), and lifespan.
3. We found that: (i) repeatability of baseline glucose level was 30%, both within and between years, (ii) having been reared in large brood and facing higher foraging costs as adult were both associated with higher baseline glucose, and (iii) survival probability decreased with increasing baseline glucose.
4. We conclude that baseline glucose level is an individual trait negatively associated with survival, which increases due to adverse environmental conditions during development and adulthood. Plasma glucose level is therefore part of the physiological processes linking environmental conditions to lifespan.

Key-words: Baseline glucose, early-life conditions, foraging cost, repeatability, survival, thrifty phenotype hypothesis, *Taenopygia guttata.*

Introduction

Circulating blood glucose is the main immediate energy source of vertebrate cells; therefore plasma glucose regulation is a key aspect of homeostasis maintenance. Nevertheless, the concentration at which plasma glucose is maintained varies between and within individuals depending on the life stage (Brzęk *et al.* 2010; Gayathri *et al.* 2004; Lill *et al.* 2002), body mass (Kaliński *et al.* 2014), age (Ferrer & Dobado-Berrios 1998; Prinzinger & Misovic 2010) and ambient fluctuations (Bairlein 1983; Remage-Healey & Romero 2000; Xue, Liang & Jiang 2012; Schradin *et al.* 2015). This variation probably has a genetic component (Svenson *et al.* 2007), combined with individual adjustment to internal and external environment (Kaliński *et al.* 2014; Glądalski *et al.* 2015). However, little is known about the causes and consequences of individual variation in baseline glucose and to what extent it can be consider an individual trait, particularly in birds.

Adverse developmental conditions have long-term effects on offspring fitness prospects in many species, including both birds (Gustafsson, Qvarnström & Sheldon 1995; de Kogel *et al.* 1997; van de Pol *et al.* 2006; Reid *et al.* 2010; Boonekamp *et al.* 2014), and mammals (Ozanne & Hales 2004; Kerr *et al.* 2007; Plard *et al.* 2015). The mechanism(s) mediating these effects have not been resolved yet, particularly in birds. However, it is evident that direct or indirect nutritional restriction (i.e. mediated through sibling competition) has long-term effects on fundamental physiological processes. Restrictive nutrient conditions during development in humans and other mammals induce higher glucose levels in adulthood (Jackson 1990; Gluckman, Hanson & Spencer 2005; Burns *et al.* 1997; Fernandez-Twin *et al.* 2005; Gardner *et al.*

2005; Fagundes *et al.* 2007), and in birds are associated with higher energy metabolism in adulthood (Verhulst, Holveck & Riebel 2006; Criscuolo *et al.* 2008; Schmidt, MacDougall-Shackleton & MacDougall-Shackleton 2012). High glucose levels in turn are associated with higher mortality risk in humans (Barr *et al.* 2007; Rao Kondapally Seshasai *et al.* 2011) and lower fledging and breeding success in birds (Kaliński *et al.* 2014; but see Glądalski *et al.* 2015). However, whether adverse developmental conditions have long-term effects on glucose homeostasis in birds or not, have to our best knowledge not been investigated yet.

Food availability, through its effect on daily work load and energy intake, is a key ecological variable that can have major consequences for health, survival and demography (Henry *et al*. 2012, Thaler *et al*. 2012). Given that physical activity consumes glucose, it is not surprising that it also modulates glucose levels. However, the effect of activity on glucose levels depends on the exercise intensity, duration and the individual's previous training. Non-migratory birds exposed to a short high-intensity flight session and non-trained humans exercised during a short bout until exhaustion increase plasma glucose during the recovery phase (birds: Viswanathan *et al.* 1987; Schwilch, Jenni & Jenni-Eiermann 1996; Abdel-Rachied *et al.* 2014; humans: Calles *et al.* 1983; Marliss *et al.* 1991). In contrast, in both birds and humans tested in a short low-intensity exercise session there was a decrease in glucose level (birds: Brackenbury & El-Sayed 1984; Brackenbury & Vincent 1988; humans: Bordel & Haase 1993). Interestingly, in humans, previous training affects the glucose response to an extensive workload session; non-trained subjects decrease plasma glucose (Ahlborg *et al.* 1974), while trained individuals increase these levels (Heath *et* *al.* 1983; Kjaer *et al.* 1986). In birds, except for studies on migratory species (i.e. Jenni-Eiermann *et al.* 2002), little is known about the effect of long-term intense workloads on glucose levels.

In this study, using adult zebra finches *Taenopygia guttata* living in outdoor aviaries, we investigated whether baseline glucose (i) is repeatable within individuals over weeks and years, (ii) is affected by ambient conditions, (iii) is related to mass and age, (iv) is affected by developmental conditions (either enlarged or reduced rearing brood size), foraging costs (either easy or hard foraging) and their interaction, and (v) is associated with survival probability. Considering the findings in other species, we predicted baseline glucose to be a repeatable trait, subject to ambient variation (e.g., temperature, season, brood size, foraging costs) and positively associated with age and mass. To evaluate the effect of brood size and foraging costs on baseline glucose, we manipulated brood size shortly after hatching to experimentally produce small and large broods, and during adulthood we exposed the birds from these brood size manipulations to a lifelong condition of either hard or easy foraging costs. We predicted birds experiencing adverse environmental conditions, either during development or in adulthood, to have higher baseline glucose levels because this appears to be associated with poorer conditions and prospects in the literature on humans and other mammals. Similarly, based on the results of research in humans and nestling birds, we predicted individuals with higher glucose levels to have lower survival probability (Barr *et al.* 2007; Rao Kondapally Seshasai *et al.* 2011; Kaliński *et al.* 2014).

Materials and methods

Birds and Housing

Birds were housed in eight single sex outdoor aviaries (L x H x W: 310 x 210 x 150 cm) located in Groningen, the Netherlands (53° 13' 0" N / 6° 33' 0" E). Each aviary contained 15 - 25 birds. At the time of glucose measurements subjects were 0.4 - 8.4 years old (mean ± s.e.m.: 3.3 ± 0.11 years). A tropical seed mixture available *ad libitum* (but see below), unrestricted access to cuttlebone, water and sand, and a supplement of 0.42 g of egg food (Bogena, Hedel, the Netherlands) per bird three times per week were provided to all subjects.

Individuals were reared in 'breeding cages' of 80 x 40 x 40 cm with a nest-box and nesting material (hay). Each breeding cage contained a single pair and unrestricted access to cuttlebone, water and sand. Egg food was provided regularly until the chicks hatched when it was removed to ensure equal diets of nestlings in broods with different numbers of young. Nests were checked several times per week and daily around the expected date of hatching.

Experimental treatments

We manipulated developmental conditions by cross-fostering all chicks to create broods that were either small (2 chicks) or large (6 chicks). Cross-fostering took place when the oldest chick of a birth nest was 4 - 5 days old. These brood sizes are within the range observed in wild zebra finches (Zann 1996). Growing up in large broods has been shown to impair growth and result in a smaller size at adulthood and shorter survival (De Kogel 1997; Tschirren *et al.* 2009), also in our colony (Briga & Verhulst 2015). Large broods thus constitute an adverse developmental environment. After nutritional independence, from the age of 35 days until approximately 120 days (when sexually mature), young were housed in larger (L x W x H: 153 x 76 x 110 cm indoor cages with up to 40 other young of the same sex and two male and two female adults (tutors for sexual imprinting) until the start of the adult treatment.

During adulthood we manipulated aviaries to have either low or high foraging costs (4 aviaries each, 2 per sex, 8 aviaries in total) as described in Koetsier and Verhulst (2011). In brief, in each aviary a food container (L x W x H: 120 x 10 x 60 cm) with 10 holes in the sides was suspended from the aviary ceiling. In the low foraging cost treatment food containers had perches beneath the holes, whereas in the high foraging cost treatment these perches were removed, forcing birds to fly from a distant perch to the food container and back for each seed. Seeds spilt by birds while feeding were collected by a duct, and hence not accessible for the birds. Birds facing high foraging costs have lower survival (Briga & Verhulst 2015) and thus high foraging costs constitute an adverse environment.

Mass and Size

Body mass was measured monthly for all the birds and the measurement closest to the blood sampling session (< 15 days) was taken for statistical analyses (birds were not weighed at sampling to minimize handling). As body mass increased through day, for statistical analysis we used the residuals of the linear regression of body mass on hour of measurement.

Structural size was measured when birds reached 100 days old and corresponds to a combined measurement of the tarsus and the head-bill length, both standard normally distributed using the equation: (standardized tarsus + standardized head-bill)/2. Residual body mass was calculated as the residuals of the linear regression of body mass on structural size.

Glucose

Blood sampling was carried out on the same population in two periods: (i) July 1st - August 9th, 2012, and (ii) August 23rd - October 2nd, 2014. Sample size was 171 birds (0.4 - 6.6 years old; mean ± s.e.m.: 3.1 ± 0.16 years) in the first sampling period (2012), and 135 birds (0.9 – 8.3 years old; mean ± s.e.m.: 3.4 ± 0.17 years) in the second one (2014). To check the repeatability of glucose levels, 57 birds were sampled twice within the same year (30 in 2012 and 27 in 2014) and 78 from the 171 birds sampled in 2012 were re-sampled in 2014. The exact same protocol was followed in both sampling periods. Before sampling, birds were taken out from their aviary and individually housed in a small box without access to food or water. The box was placed in a dark room for 30 minutes together with two other boxes containing birds from the same aviary. The aim of this standard procedure is to yield baseline glucose values, independent of recent food consumption. A pilot study, conducted using a different sample of birds of the same colony, showed that intra-individual glucose levels between 30 and 60 minutes after the capture stay in a relative stable state (Montoya *et al.* unpublished data). Consequently, to reduce stress associated to alimentary, social and motor restriction we used the shortest waiting time within this interval. After 30 minutes a 70 µL blood sample was taken from the brachial vein and collected in heparinized capillaries.

Immediately after sampling, blood was diluted 30x in a heparin (500 IU/mL) - 0.01% EDTA solution and frozen until glucose measurement.

For the measurement of whole blood glucose levels we performed the Hoffman's ferricyanide method using a Thechnicon autoanalyzer (Beckman Coulter LX20PRO). All blood samples were analyzed in duplicate obtaining an ICC of 72.4% (n = 137 measurements, 95% C.I. 64.5, 79%).

Statistical analyses

All statistical analyses were performed using R version 3.1.2 (R core team 2014). Glucose values were ln transformed prior to analysis to obtain a normal distribution, and mean centered by year when year was not included in the model as fixed factor. To explore the association of glucose levels with environmental and individual variables we fitted linear mixed models with the function lmer in the package lme4 (Bates *et al.* 2015). To control for stochastic variation associated to sampling session we included sampling *day* (starting from 1 on a year's first day of sampling) as a random factor in all models. To correct for daily climatic variation, we used sampling *date*, which is a continuous variable and refers to day number in the year when the sample was taken (counting on from January 1st). To account for repeated measures of one individual, we included individual identity as random factor in these models. Survival analysis was performed fitting a Cox proportional hazards (CPH) (coxme package; Therneau 2012). Because age at sampling is not proportional, we stratified sampling age into two groups of equal range. Repeated measures per individual were accounted for by including individual identity as a random term, nested in aviary. All survival analyses were checked for the proportionality assumption, using Schoenfeld residuals and with the *'cox.zph'* function. Linearity and influential datapoints were checked with Martingale and deviance residuals respectively.

Results

Repeatability

We calculated the repeatability of glucose level on two levels: within years and between years. Data for the repeatability estimates were collected in two sessions in each of the two years (i.e. four sessions in total). Because average glucose levels differed between these four sessions ($P < 0.0001$), we used deviations from the session average for the repeatability calculations. Repeatability (intra-class correlation coefficient) within years was 29.7% (Fig. 1a; n = 57 individuals, 95% C.I.: 2.7- 56.7%). Repeatability between years was almost identical, at 27.4% (Fig. 1b; n = 81 individuals, 95% C.I.: 4.7- 50.1%). Thus glucose level is an individual characteristic of zebra finches, even over a period of years.

Is glucose level associated with environmental variables?

Before examining the association between baseline glucose level with experimental treatments, age and survival, we evaluated whether glucose levels were related to year of sampling and ambient variables (time of the day, time of the day squared, day length and ambient temperature). Glucose level was almost 20% lower during the first of the two study years (Fig. 2a; $F_{229.51} = 33.18$, $P < 0.001$; first year mean ± s.e.m.: 12.98 ± 0.10 mM; second year: 16.03 ±

0.18 mM). Furthermore, glucose levels decreased with higher temperatures and day length (Table 1; Fig. 2). To increase statistical power we included sampling day as random factor in models of subsequent sections, which summarizes variation associated with temperature, day length and possible additional unidentified sources of variation between days.

Sex, age, size and body mass

There was a non-significant tendency for males to have higher glucose than females (effect of sex added to minimal model in Table 1; males had 1.50% more glucose than females; $F_{1, 217.18} = 3.23$, $P = 0.07$). However, this factor was excluded from models in subsequent sections, as it never explained a significant portion of the variation as a main factor nor in interactions ($p > 0.10$).

Aging has been associated with a decline in baseline metabolic rate in our study species (e.g. Moe *et al.* 2009) but we found no association between age (or age squared) and glucose levels (Table 3). Early or adult life conditions could also differentially affect glucose levels depending on age, but interactions of age with these factors were not statistically significant (Table 3). Similarly, there was not significant interaction between age and sex ($F_{1, 310.86} = 0.26$, $P = 0.61$).

There is usually a strong positive association between energy turnover and body mass, and we therefore evaluated the association between glucose level and body mass. Variation in body mass arises through variation in structural body size, and because individuals of a given body size can have different body composition e.g. muscle mass and energy reserves. We thus separated effects of size and body composition by splitting mass in two components: structural body size, and residuals from the regression of mass on body size. We explored for an association between glucose levels and body mass or size, adding these variables one by one to a model with only sampling day and bird identity (random effects). Neither body mass ($F_{1,305.02} = 0.40$, $P = 0.52$), residual body mass ($F_{1,295.7} = 0.24$, $P = 0.62$) or structural size ($F_{1,197.85} = 0.23$, $P = 0.63$) were significantly associated with glucose level. Similarly, there was no significant interaction of sex with body mass ($F_{1,316.06} = 2.79$, $P = 0.10$), residual body mass ($F_{1,291.41} = 3.54$, $P = 0.06$) or structural size ($F_{1,199.08} = 0.11$, $P = 0.74$). We note though that males with higher residual body mass tended to have higher baseline glucose ($\beta = 0.14 \pm 0.08$), whereas in females the opposite association was found.

Manipulated environmental conditions

Considering that glucose level was a repeatable trait, and thus an individual characteristic, we tested whether it was affected by our (permanent) experimental manipulations. Because glucose level differed significantly between years, even when controlling for the environmental variables (Table 1), we used glucose values mean centered by year in these analyses (i.e. observed value – the year specific mean value) and included sampling day as random effect to control for environmental variation within years. Glucose level was higher in birds reared in large broods, and, similarly, in birds exposed to higher foraging cost (Table 2, Fig. 3). We did not find an interaction between early and adult environmental conditions with respect to glucose level ($F_{1,207.78} = 0.04$, $P = 0.84$; Table 2). One could speculate that individual observations within aviaries are not statistically independent, but adding aviary identity as random effect to the model did neither explain significant variation (REML, $P = 0.22$), nor cause substantial changes in the model, indicating statistical independence of individual measurements within aviaries.

Survival

By January 2016, 120 of the 170 birds sampled in 2012 and 56 of the 134 birds sampled in 2014 had died. Note that because some birds were sampled twice, so of the 225 birds sampled, 176 had died. To correct the glucose measurement for variation associated to sampling date and ambient temperature in the survival analyses, we used the residuals of the linear regression of sampling date and ambient temperature on baseline glucose. Birds with the highest levels of glucose had lower survival probability (Fig. 4), even when controlling for variables previously associated with mortality in this laboratory population such as age, rearing brood size and foraging treatment (Table 4). We also tested for a quadratic survival effect of glucose level, but this was not significant ($\beta = -0.002 \pm 0.012$, $P = 0.88$).

Discussion

Individual variation in glucose level was repeatable, and repeatability within and between years was almost indistinguishable at 30 and 27% respectively. These values are slightly lower than values reported for zebra finches for some other physiological traits (Careau, Buttemer & Buchanan 2014), and lower when compared to within months repeatability in non-insulin dependent diabetic humans, but higher when compared to insulin dependent patients (Pecoraro *et* *al.* 1986; Temple, Bar-Or & Riddell 1995). Significant repeatability of plasma glucose implies consistent variation between individuals in the level at which plasma glucose is regulated. However, to be affected by natural selection, the timeframe in which this trait is repeatable should be long enough to associate with individual variation in fecundity or survival. In this study, baseline glucose was found to be equally repeatable within a period of two months and two years, satisfying this condition. Moreover, high plasma glucose level was negatively linked to survival probability (Fig. 4), suggesting this trait to be a target of selection. Whether selection is translated into an evolutionary response will depend among other things on the heritability of plasma glucose level, which remains to be estimated in our study system. Other physiological traits in birds, also associated with energy expenditure, such as metabolic rate (Rønning, Moe & Bech 2005; Nespolo & Franco 2007) and baseline corticosterone (Angelier *et al.* 2010; Small & Schoech 2015; B. Jimeno unpublished data), have also shown to be repeatable through the individual's life. This suggests that the level of energy metabolism more generally may be a target of natural selection.

Birds reared in enlarged broods had 11% higher baseline glucose than birds raised in reduced broods (Fig. 3). This finding is to our best knowledge new for birds, but consistent with the proposed long-term effects of early-life adverse conditions on glucose homeostasis in adult mammals (Desai *et al.* 1997; Gardner *et al.* 2005; Fagundes *et al.* 2007), and with previous findings regarding the effect of growing up in a large brood on adult metabolic efficiency in zebra finches (Verhulst *et al.* 2006). Additionally, birds living in hard foraging conditions during adulthood had 19% higher baseline glucose than birds in easy foraging conditions (Fig. 3). Birds forced to fly to obtain their food could have maintained high glucose concentration due to two processes. Firstly, they may rely on glucose to fuel successive foraging bouts that are more demanding than foraging in the easy foraging conditions. Secondly, hard foraging conditions may have worked as endurance training, and consequently, birds in this treatment might depend on other fuel sources different to glucose, maintaining glucose levels high, as was shown to be the case in trained humans (Heath *et al.* 1983; Kjaer *et al.* 1986). In either case our results indicate that developmental and adult adverse nutritional conditions affect glucose metabolism in adulthood.

The effect of harsh foraging conditions on glucose levels was independent of developmental conditions (brood size), because early and adult life manipulations did not significantly interact to affect glucose levels in adulthood (Table 2). This contrasts with expectations based on the thrifty phenotype hypothesis, which proposes that allowing high glucose levels in the blood stream when dealing with nutrient restrictive developmental conditions is an adaptive response when adult nutritional environment matches with early-life conditions (Hales & Baker 1992, 2001; Gluckman *et al.* 2005: Hanson & Gluckman 2014). However, a thrifty strategy is hypothesized to be maladaptive when the adult environment is nutrient-rich, contrasting with developmental conditions, resulting in detrimental high glucose levels (Langley-Evans 2006). Our results are inconsistent with this hypothesis because effects of developmental and adult conditions were additive (i.e. did not interact). We note however that our finding is in agreement with the lack of broad support for the match-mismatch analysis emerging from a recent meta-analysis, summarizing effects on a wide range of end-points (Uller, Nakagawa & English 2013).

High baseline glucose level was associated with higher mortality (Fig. 4). This result is consistent with epidemiological studies of human populations, where elevated fasting glucose increases all causes mortality probability after age 25 years (Barr *et al.* 2007), but we are not aware of studies in other species testing for this relationship. It is worth noting that the association between glucose level and mortality was independent of age and the experimental manipulations, and thus the mortality effect cannot be attributed to these potential confounds. Our findings indicate that there can be selection against high glucose levels, and that developmental and adult life conditions contribute to the expression of this negatively selected trait.

We found no relationship between age and glucose level in our cross-sectional analysis, but a positive relationship between baseline glucose and mortality. Paradoxically, this combination of findings indicates that plasma glucose levels actually do increase with age within individuals, because, in a cross-sectional analysis, otherwise selective disappearance of high glucose individuals would generate a negative association between age and plasma glucose. We do stress however that a longitudinal study is required to confirm this interpretation.

Given that elevated glucose increases mortality probability, our results raise the question as to why individuals regulate their plasma glucose at high levels. High baseline glucose levels were shown to increase performance of physical and cognitive demanding activities (Rodríguez, DiMarco & Langley 2009; Gilsenan, de Bruin & Dye 2009), and the short-term benefits of having high glucose levels might thereby outweigh any long-term survival cost. In this way, plasma glucose level may more generally reflect where individuals are on the slow-fast life history continuum. Studies on the association of plasma glucose with other life history traits, in particular reproductive investment, are required to verify this hypothesis.

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Data accessibility

All data used in this manuscript will be made available upon acceptance.

Competing interests

The authors declare no competing interests.

Author contributions

SM performed bird sampling and glucose measurements in 2012, and BM and BJ in 2014. BM, MB and SV analyzed data. MB and BJ ran the long-term experiment designed by SV, and collected survival, body mass and structural size data. BM, SV and MB wrote the first draft of the manuscript and all authors contributed to later versions.

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Figures

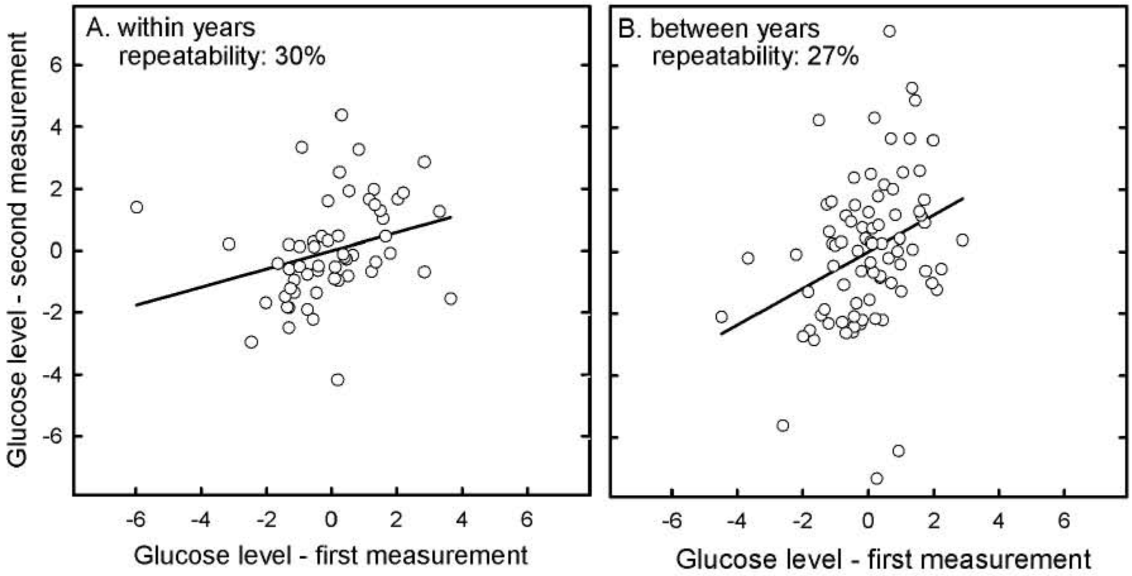

Figure 1. Individual repeatability of baseline glucose levels (mM) within years (A) and between years (B). Plotted data show second measurement plotted against the first measurement in both cases. Data points are deviations from the mean glucose level in each of the measurement sessions to account for within and between year variations in average glucose level.

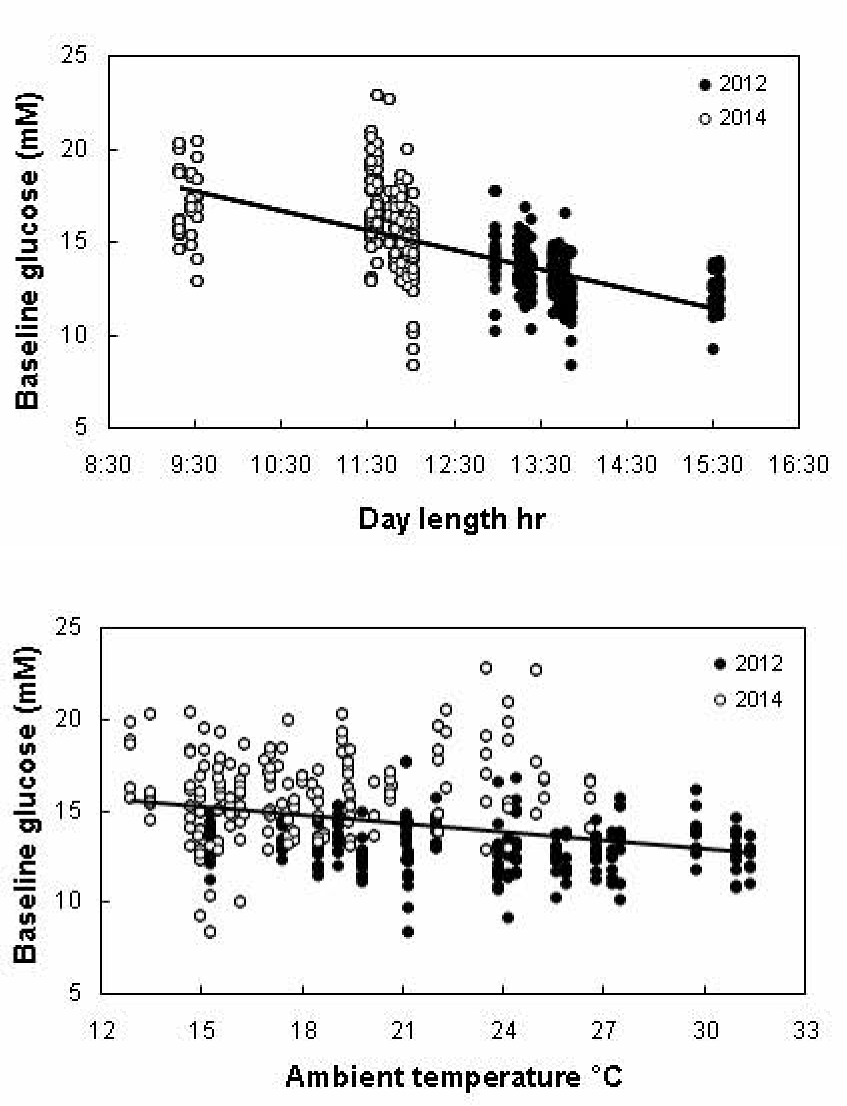

Figure 2. Baseline glucose level (mM) in relation to day length (A) and ambient temperature (B). Closed circles correspond to birds sampled in 2012 and open circles to birds sampled in 2014.

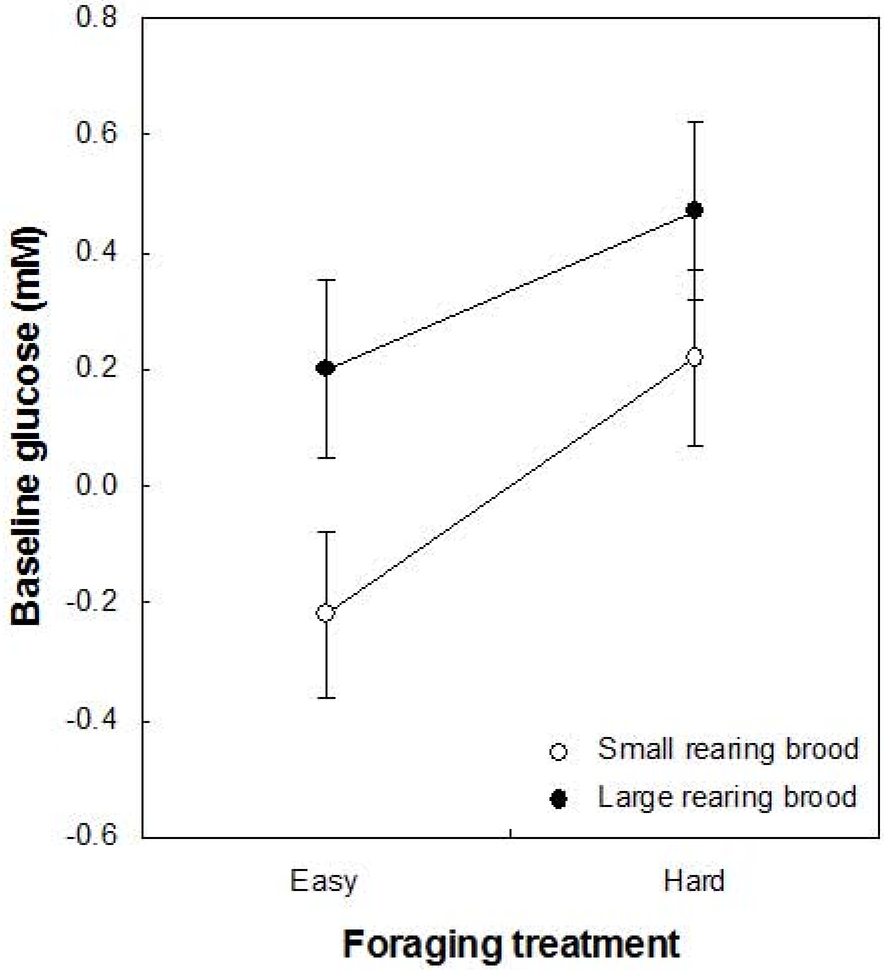

Figure 3. Manipulated environmental conditions (rearing brood size and foraging treatment) and model estimates of baseline glucose (mM ± s.e.m.; data mean centered by year). Open circles correspond to birds reared in small broods (2 chicks), and closed circles correspond to birds reared in large broods (6 chicks).

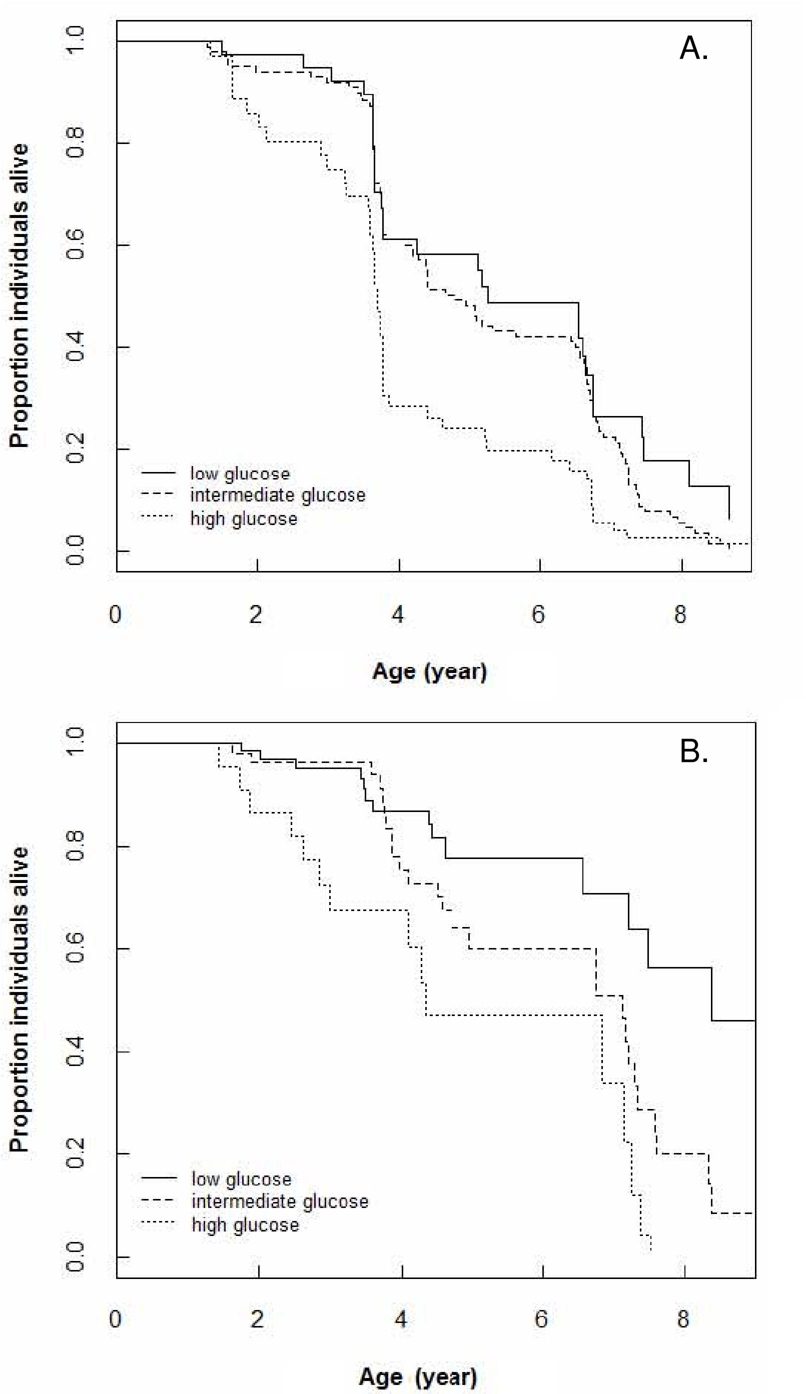

Figure 4. Plasma baseline glucose level and survival. Panel A shows birds sampled in 2012 and panel B individuals sampled in 2014. In both panels, solid line corresponds to low glucose level (mM, lower quartile), dashed line to intermediate glucose (two middle quartiles pooled), and dotted line to high glucose (upper quartile). Note that this grouping is for illustrative purposes only; glucose level was treated as a continuous variable in the analysis.

Tables

Table 1. Plasma baseline glucose level (mM) and its association with environmental factors.

Fixed effects	**Coefficient (± s.e.)**	**Den DF**	***F***	***P***
Temperature	0.004 ± 0.001	329.77	6.71	0.010
Day length	-1.15 ± 0.18	262.87	42.81	<0.001
Year (relative to 2012)	0.11 ± 0.02	229.51	33.18	<0.001
Rejected terms				
Time of the day	-0.05 ± 0.08	336.89	0.41	0.52
Time of the day^2	-0.13 ± 0.54	291.26	0.059	0.81
Random effects	**Variance**	**Standard deviation**		
Bird identity	0.29	0.54		

* For analyses glucose levels were ln transformed, n = 227 individuals.

Table 2. Plasma baseline glucose level (mM) and its association with manipulated developmental and adult conditions.

Fixed effects	**Coefficient (± s.e.)**	**Den DF**	***F***	***P***
Foraging treatment (Hard)	0.24 ± 0.10	200.34	5.58	0.02
Rearing brood size (Large)	0.22 ± 0.10	206.88	4.50	0.04
Rejected terms				
Foraging T x Rearing BS	-	207.78	0.04	0.84
Random effects	**Variance**	**Standard deviation**		
Bird identity	0.27	0.52		
Sampling day	0.19	0.43		

* For analyses glucose levels were mean centered by year, n = 227 individuals.

Table 3. Relationship between age and glucose level (mM).

Variable (Fixed effect)	**Coefficient (± s.e.)**	**Den DF**	***F***	***P***
Foraging treatment (Hard)	0.24 ± 0.10	200.34	5.58	0.02
Rearing brood size (Large)	0.22 ± 0.11	206.88	4.50	0.04
Rejected terms				
Age	0.01 ± 0.02	267.90	0.22	0.64
Age^2	-0.004 ± 0.01	256.12	0.14	0.70
Foraging treatment (H) * Age	-0.06 ± 0.05	309.03	1.72	0.19
Rearing brood size (L) * Age	-0.02 ± 0.05	315.25	0.13	0.71
Random effects	**Variance**	**Standard deviation**		
Bird identity	0.27	0.52		
Sampling day	0.19	0.43		

* For analyses glucose levels were mean centered by year. Models controlled by environmental variation associated to sampling date and experimental treatments, n = 227 individuals.

Table 4. Relation between residual glucose (mM) and mortality probability, fitted with a Cox proportional hazards model.

Fixed effects	**Exp(coef) ± s.e.**	***z***	***P***
Residual glucose	1.09 ± 0.04	2.15	0.032
Rearing Brood Size (L)	0.96 ± 0.04	-1.14	0.25
Foraging Treatment (H)	1.33 ± 0.26	1.07	0.29
Random effects	**Variance**	**Standard deviation**	
(Bird identity)Aviary	1.35E-12	1.16E-6	

* For the cox proportional hazards model residual glucose was calculated from the linear regression of sampling day and ambient temperature on baseline glucose n = 304 samples on 225 individuals of which 176 died.