Universidad Autónoma de Tlaxcala

Posgrado en Ciencias Biológicas

Efectos maternos pre- y post-cigóticos:

El papel de las hormonas y la conducta

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Yetzi América Hernández Martínez

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Tlaxcala, Tlax.   Enero, 2023

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P R E S E N T E

Los abajo firmantes, miembros del jurado evaluador del proyecto de tesis que **Yetzi América Hernández Martínez** realiza para la obtención del grado de **Doctora en Ciencias Biológicas**, expresamos que, habiendo revisado la versión final del documento de tesis, damos la aprobación para que ésta sea impresa y defendida en el examen correspondiente. El título que llevará es **"Efectos maternos pre- y post-cigóticos: El papel de las hormonas y la conducta"**.

Sin otro particular, aprovechamos para enviarle un cordial saludo.

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TLAXCALA, TLAX., A 29 DE NOVIEMBRE DEL 2022

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A Adriana, Pepe, Uriel, Isamar e Ivan

Con todo mi amor

RESUMEN GENERAL

Durante la vida temprana se consolidan estructuras corporales y funciones vitales, por lo que las perturbaciones experimentadas en esta etapa, pueden producir cambios fenotípicos a corto y largo plazo en la morfología, conducta y fisiología de los individuos. Tanto el ambiente donde el individuo nace y crece, como aquél experimentado por los padres en diferentes etapas de su vida, puede afectar el fenotipo y la adecuación de los individuos. A la influencia que el fenotipo materno o las condiciones ambientales de la madre puede tener sobre el fenotipo de las crías se le denomina efecto materno. Unos de los efectos maternos más estudiados son aquellos mediados por la acción de hormonas maternas de forma previa o posterior a la fecundación (*i. e.* efectos maternos pre- y post-cigóticos, respectivamente). Se ha sugerido que los efectos maternos pueden tener un valor adaptativo al favorecer a la descendencia en determinados entornos; sin embargo, también podrían tener un impacto negativo sobre la adecuación. Por lo que en esta tesis se investigaron efectos maternos mediados por hormonas pre- y post-cigóticos en dos modelos aviares. En el capítulo 1, se evalúo si la competencia materna por recursos clave para el evento reproductivo favorece la ocurrencia de efectos maternos mediados por hormonas en el azulejo de garganta azul (*Sialia mexicana*), afectando el desarrollo y supervivencia de la descendencia. Se encontró que las hembras experimentales, es decir, aquellas que se reproducen en sitios en los cuales se redujo experimentalmente la disponibilidad de cajas nido y donde se presentó un señuelo de un competidor potencial, no modificaron los niveles de testosterona en la yema. Sin embargo, en comparación con las crías del grupo control, las crías de las hembras experimentales fueron más ligeras al inicio del desarrollo, mostraron una mayor ganancia de masa durante la fase de crecimiento rápido, un menor crecimiento de ulna durante la fase de crecimiento lento, y tarsos más largos (en las crías macho) a la edad de 15 días. Así mismo, las crías experimentales tuvieron tasas respiratorias (un indicador de la conducta exploratoria) menores que las crías controles, y aunque las diferencias no fueron estadísticamente significativas, la probabilidad de supervivencia al emplumado fue 33% menor en las crías experimentales que en las controles. Estos resultados contrastan con los de estudios realizados previamente en poblaciones norteñas de la misma especie, donde una menor disponibilidad cajas nidos se relaciona con incrementos en los niveles de testosterona depositados en la yema del

huevo. Esta discrepancia entre estudios probablemente sugiere que los mecanismos que subyacen a los efectos maternos podrían variar en función del contexto ecológico, y discutimos la posibilidad de que los efectos del tratamiento experimental en crecimiento y supervivencia reflejen cambios en el comportamiento parental. En el capítulo 2, se evaluó experimentalmente si la competencia por cavidades de anidación afectó los niveles de corticosterona y daño oxidante en lípidos en las crías de azulejo a los 3 días de edad (la medida más cercana a la eclosión) y si, estas posibles modificaciones, se vincularon con la trayectoria de crecimiento y sexo de ellas. Se encontró que los niveles de corticosterona y daño oxidante en lípidos circulantes no se relacionan con la competencia por cavidades de anidación. Sin embargo, la concentración de corticosterona y de daño en lípidos en sangre se relacionaron negativamente con el crecimiento estructural, únicamente en las crías macho. Este resultado sugiere que mantener altos niveles de corticosterona pueden tener repercusiones negativas sobre el crecimiento y la condición durante la vida temprana (*i. e.* CORT–Fitness Hypothesis), pero estos efectos difieren entre los sexos. En el capítulo 3, se evaluó si alteraciones en la temperatura óptima de incubación, potencialmente producto de cambios en la conducta parental, pueden impactar el fenotipo de las crías debido a sus efectos en el metabolismo de las hormonas maternas depositadas en el huevo. Para evaluar estas hipótesis, se usó como modelo de estudio a la codorniz japonesa (*Coturnix japonica*) y por medio de un diseño factorial 2 x 2, se manipuló experimentalmente la concentración de testosterona en la yema y la temperatura de incubación (*i. e.* 36° C *vs.* 38 °C). Se encontró que los huevos expuestos a alta concentración de testosterona e incubación a 36° C incrementaron la concentración de 5α-dihidrotestosterona. Las concentraciones de testosterona y estradiol no fueron afectadas por la inyección de testosterona ni la temperatura de incubación. Si las condiciones ambientales afectan el comportamiento de incubación de la hembra, podría esperarse que un cambio en la conducta de incubación repercuta en la temperatura a la que son expuestos los embriones en desarrollo y, consecuentemente, modifiquen en respuesta a dichos cambios ambientales las hormonas que fueron depositadas en la yema y a las que estará expuesta la cría. Sin embargo, poco se sabe sobre las consecuencias que esto podría tener a corto y largo plazo sobre el fenotipo de la descendencia. En conjunto, los resultados de esta tesis sugieren que las interacciones sociales experimentadas por la madre (*e. g.* competencia por cavidades), y los cambios que estas producen en su comportamiento (*e.*

g. incubación), podrían promover la acción de las hormonas esteroides y jugar un rol importante en la determinación del fenotipo de las crías. Aunque este trabajo sugiere que las condiciones ambientales experimentadas por la madre pueden afectar el fenotipo de la cría a corto plazo, aún son necesarios trabajos que evalúen el efecto potencial de las condiciones pre- y post-natales a largo plazo, reproducción y supervivencia en la adultez, con el fin de evaluar su potencial adaptativo.

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INTRODUCCIÓN GENERAL

Efectos maternos mediados por hormonas

El fenotipo es el conjunto de características de los individuos que resulta de la variación genética y su interacción con la variación ambiental. La vida temprana, es decir el período que comprende desde la fecundación hasta la madurez sexual, ha sido considerada una ventana temporal crítica para el desarrollo del fenotipo de los organismos, ya que las perturbaciones ambientales experimentadas durante este periodo sensible pueden ejercer una influencia importante en estructuras y procesos fisiológicos en consolidación (Lindström 1999). Enfrentar condiciones adversas durante el desarrollo temprano puede tener consecuencias a corto y largo plazo sobre la morfología, conducta y fisiología de los individuos, y por consiguiente en su adecuación (Metcalfe y Monaghan 2001, Monaghan 2008). Tradicionalmente, el ambiente temprano ha sido considerado como el medio físico y social donde el individuo nace y crece, pero numerosos estudios han mostrado que el ambiente experimentado por los padres en diferentes etapas de su vida podría afectar indirectamente el fenotipo de la descendencia (Mousseau y Fox 1998, Monaghan 2008). La influencia del fenotipo o de las condiciones del ambiente experimentado por los padres sobre el fenotipo de los hijos es definida como efecto parental (Uller 2008). El ambiente de la madre, en particular, puede influir de manera muy importante sobre el fenotipo y la adecuación de la descendencia (*i. e.* efectos maternos, Mousseau y Fox 1998), y estos efectos maternos suelen ser mediados por hormonas (Groothuis et al. 2019). Dicha influencia materna puede ocurrir de forma previa o posterior a la fecundación (*i. e.* efectos maternos pre- y post-cigóticos, Lombardi 1996), por lo que el contexto ecológico experimentado por la hembra podría modificar el desarrollo del crío mediante cambios en la asignación de recursos para el desarrollo de la progenie como la exposición a hormonas maternas durante el desarrollo embrionario o a través de modificaciones en el cuidado parental que resulten en cambios fisiológicos en la descendencia.

Efectos maternos pre-cigóticos: Deposición de hormonas esteroides en el huevo

Las hormonas esteroides han sido consideradas como los principales inductores de los efectos maternos mediados por hormonas, siendo los andrógenos los más estudiados (Moore y Johnston 2008). Las hormonas esteroides son derivados del colesterol y producidos mediante una serie de reacciones de hidroxilación y eliminación (parcial o total) de la cadena lateral de carbonos del colesterol obtenido de las lipoproteínas del plasma (Durán et al. 2011, Figura 1a). Una vez que han sido sintetizados los esteroides, son transportados por el torrente sanguíneo, mediante proteínas de transporte (*e. g.* globulina de unión a corticosterona), hacia el tejido diana donde se unen a receptores específicos para ejercer su acción (Nelson y Cox 2017). Dos tipos de hormonas esteroides pueden ser generados: los corticoides y las hormonas sexuales. En el primer grupo se encuentran los mineralocorticoides (*e. g.* aldosterona) y los glucocorticoides (*e. g.* corticosterona); mientras que, las hormonas sexuales comprenden a los andrógenos (*e. g.* testosterona, androstenediona y la 5α-dihidrotestosterona) y los estrógenos (*e. g.* estradiol; Nelson y Cox 2017).

Las hormonas esteroides desempeñan un papel importante en la reproducción femenina. La síntesis de las hormonas sexuales está controlada por el eje hipotálamo-pituitaria-gonadal (Nelson y Cox 2017). Los andrógenos estimulan la síntesis de la albúmina en el magnum (Johnson 2015). La testosterona es la hormona sexual producida principalmente en las gónadas masculinas; sin embargo, este andrógeno también es secretado por las hembras en los folículos del ovario, de manera particular, en la teca interna (*i. e.* la capa de células más próxima que rodea a la yema, Groothuis y Schwabl 2008, Figura 1b) y, en menor medida, en las glándulas adrenales (Johnson 2015). Tanto en machos como en hembras, la testosterona puede ser convertida a 5α-dihidrotestosterona, su metabolito más activo, además de ser el precursor de los estrógenos (Ketterson et al. 2005). Los estrógenos, como el estradiol, participan en la formación de la yema durante la maduración folicular y la ovulación (Johnson 2015), éstos son sintetizados en la teca externa del folículo. Por otro lado, los corticoides son regulados por el eje hipotálamo-pituitaria-adrenal, siendo la corticosterona el principal glucocorticoide secretado en aves. La corticosterona es secretada de forma previa a la ovulación regulando la secreción de hormona luteinizante (responsable de la maduración del ovario y de la ovulación), durante la puesta y cuidado parental de la descendencia (Carsia 2015). Además, la concentración de este

glucocorticoide se encuentra relacionada con requerimientos energéticos (Landys et al. 2006) suscitados durante eventos predecibles, como el crecimiento, la migración o la reproducción, ya que favorece la movilización de la energía requerida para el desarrollo de los rasgos necesarios en dichos procesos. Asimismo, los niveles de corticosterona incrementan ante situaciones impredecibles e incontrolables (Wingfield y Romero 2010) lo que permite la reasignación de recursos energéticos a funciones vinculadas con la supervivencia inmediata ante una situación crítica.
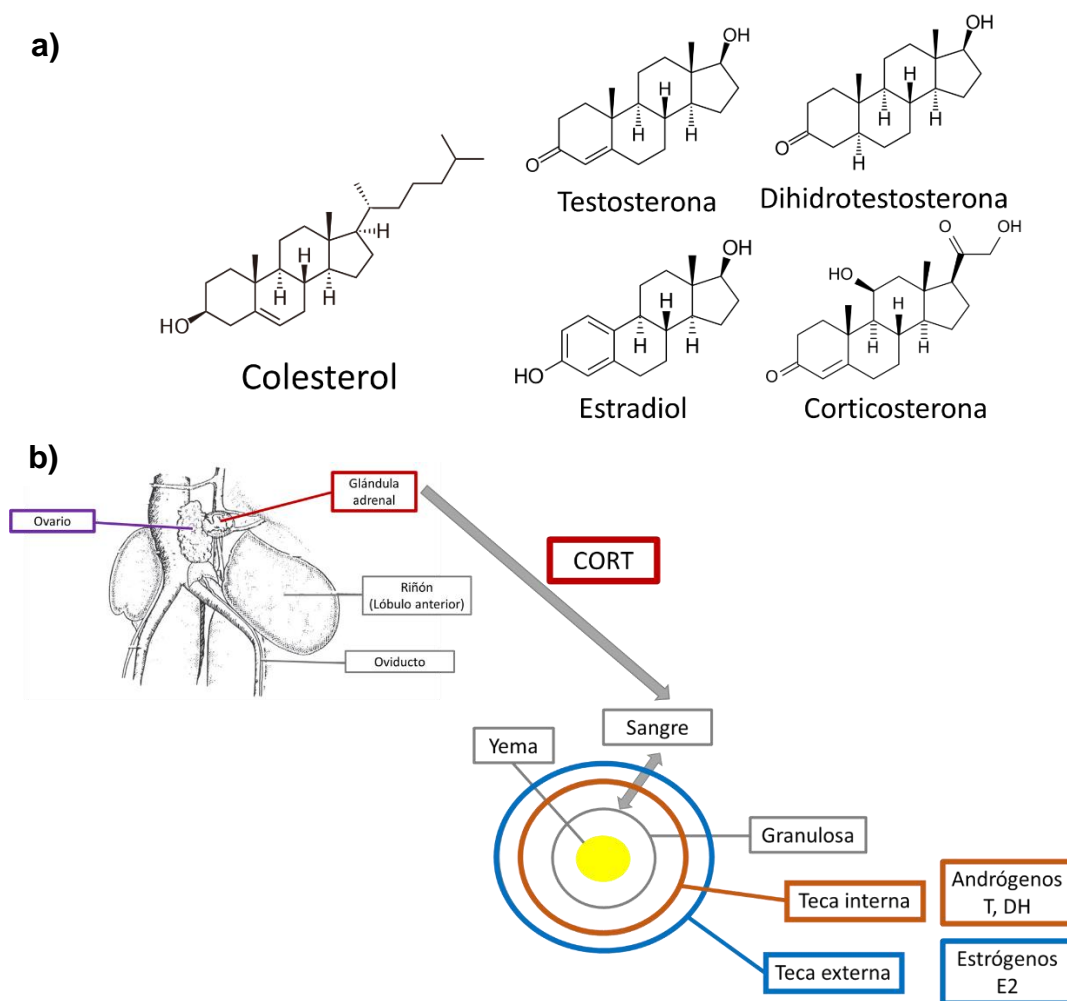
Figura 1. a) Estructura química del colesterol, testosterona, dihidrotestosterona, estradiol y corticosterona (imágenes tomadas de la red, https://uniiquim.iquimica.unam.mx). b) Diagrama de la localización del ovario y glándula adrenal aviar (modificado de Carsia 2015), así como de las capas celulares del folículo del ovario mostrando la producción y transferencia de las hormonas esteroides a la yema (modificado de Groothuis y Schwabl 2008). T: Testosterona, DHT: 5α-dihidrotestosterona, E2: Estradiol.

Las hormonas sexuales y los glucocorticoides pueden ser depositados en los diferentes componentes del huevo durante su producción (Groothuis y Schwal 2008), tanto en el cascarón (*e. g.* Qasimi et al. 2018), como en el albumen (*e. g.* Hayward y Wingfield 2004, Rubolini et al. 2005, Downing y Bryden 2008, Pitk et al. 2012) y la yema (*e. g.* Pike y Petrie 2005, Navara et al. 2006b, Coslovsky et al. 2012). Sin embargo, pese a que el albumen es producido en unas cuantas horas durante su paso por el magnum (Burley y Vadehra 1989) y que el volumen de este componente es mucho mayor en comparación con el de la yema, la propiedad lipofílica de las hormonas esteroides podría permitir una mayor deposición de éstas en el medio graso de la yema en comparación con el medio acuoso del albumen (Groothuis y Schwal 2008). Asimismo, en la yema es donde ocurre el desarrollo del embrión (Johnson 2015), por lo que los efectos de la exposición hormonal durante el desarrollo temprano han sido ampliamente estudiados como consecuencia de la deposición de hormonas maternas en la yema.

Existen tres hipótesis principales sobre los mecanismos que subyacen a la deposición de hormonas de origen materno en el huevo. La *hipótesis del epifenómeno fisiológico* (PEH, por sus siglas en inglés), sugiere que la concentración de hormonas en la yema de huevo es un subproducto de la síntesis de hormonas relacionado con los procesos fisiológicos de la hembra. Por lo cual, se propone que no existe un mecanismo independiente que regule los niveles hormonales circulantes en la madre y el huevo y, en consecuencia, se espera una relación positiva entre ambas concentraciones de hormonas (*e. g.* Whittingham y Schwabl 2002, Jawor et al. 2007). Por el contrario, la *hipótesis de la distribución flexible* sugiere que la hembra puede incrementar la deposición de hormonas en la yema a expensas de sus propios niveles circulantes, por lo que se predice una asociación negativa entre la concentración de la hormona en el torrente sanguíneo de la hembra y en la yema (*e. g.* Mazuc et al. 2003, Navara et al. 2006b, Lattin et al. 2016). Finalmente, la *hipótesis de la regulación independiente* propone que dos mecanismos independientes podrían regular la concentración de hormonas esteroides en el huevo y el torrente sanguíneo de la hembra. De acuerdo con esta hipótesis, no se esperaría una relación entre los niveles hormonales de la madre y los depositados en la yema (*e. g.* Langen et al. 2017, Bentz et al. 2018). La PEH ha sido considerada como la propuesta más parsimoniosa (Groothuis y Schawbl 2008), no obstante, los estudios que lo han puesto a prueba no proveen resultados consistentes, ya que no en todos los casos se ha encontrado la relación positiva esperada entre

los niveles de las hormonas esteroides circulantes en la hembra y los depositados en el huevo (Williams et al. 2004).

Estudios empíricos realizados en diferentes especies en los últimos treinta años, han mostrado que la concentración de hormonas esteroides en el huevo puede modificarse en respuesta a factores experimentados por la madre durante la puesta, como un aumento en la carga parasitaria, una elevación del riesgo de depredación, una reducción en disponibilidad de recursos, presencia de huevos de otras hembras (*i. e.* parásitos de puesta), así como un incremento en la competencia con conespecíficos y heterospecíficos por recursos clave, como los sitios de anidación (Tabla 1). Por ejemplo, los niveles de competencia y agresión a los que la hembra está expuesta antes de la puesta se han asociado con un incremento en la concentración de hormonas en el huevo (Bentz et al. 2016a). Sin embargo, las discrepancias en el patrón de deposición hormonal, es decir el incremento o la disminución en la transferencia de hormonas de origen materno al huevo, puede depender de las disyuntivas entre los costos y los beneficios que podría experimentar el embrión bajo un determinado contexto ambiental (Groothuis et al. 2019, 2020).

Tabla 1. Estudios que evalúan la dirección en el cambio de la concentración de hormonas (T: Testosterona, A4: Androstenediona, DHT: Dihidrotestosterona, CORT: Corticosterona, E2: Estradiol) depositadas en el huevo con base a estímulos ambientales experimentados por la madre. +: Positiva, -: Negativa, 0: Sin diferencias.

Referencia	Tipo de estudio	Especie	Hormona	Estímulo	Dirección del estímulo	Dirección de la concentración hormonal en la yema
Schwabl 1997	Experimental	*Passer domesticus*	T	Densidad reproductiva	+	+
Reed y Vleck 2001	Experimental	*Fulica americana*	T	Densidad reproductiva	+	+
Groothuis 2002	Correlativo	*Larus ridibundus*	T	Densidad reproductiva	-	+
Whittingham y Schwabl 2002	Experimental	*Tachycineta bicolor*	T	Invasión territorial/Agresión	+	+
Mazuc et al. 2003	Experimental	*Passer domesticus*	T	Invasión territorial/Agresión	+	+
Pilz y Smith 2004	Correlativo	*Sturnus vulgaris*	T/A4	Densidad reproductiva	+	+
Tschirren et al. 2004	Experimental	*Parus major*	A4	Carga parasitaria	-	+
Tschirren et al. 2004	Experimental	*Parus major*	T	Carga parasitaria	-	+
Tschirren et al. 2004	Experimental	*Parus major*	DHT	Carga parasitaria	-	0
Verboven et al. 2005	Experimental	*Larus fuscus*	T	Invasión territorial/Agresión	+	0
Pike y Petrie 2005	Experimental	*Pavo cristatus*	CORT	Atractivo del macho	-	+
Pike y Petrie 2005	Experimental	*Pavo cristatus*	T	Atractivo del macho	-	-
Pike y Petrie 2005	Experimental	*Pavo cristatus*	E2	Atractivo del macho	-	0

Saino et al. 2005	Experimental	*Hirundo rustica*	CORT	Estrés materno	+	+
Cariello et al. 2006	Experimental	*Guira guira*	A4	Competencia	+	+
Cariello et al. 2006	Experimental	*Guira guira*	T/DHT	Competencia	+	0
Navara et al. 2006b	Experimental	*Sialia sialis*	A4	Invasión territorial/Agresión	+	+
Navara et al. 2006b	Experimental	*Sialia sialis*	T	Invasión territorial/Agresión	+	+
Navara et al. 2006b	Experimental	*Sialia sialis*	E2	Invasión territorial/Agresión	+	0
Navara et al. 2006b	Experimental	*Sialia sialis*	CORT	Invasión territorial/Agresión	+	0
Eising et al. 2007	Experimental	*Sturnus vulgaris*	T	Densidad reproductiva	+	+
Eising et al. 2007	Experimental	*Sturnus vulgaris*	DHT	Densidad reproductiva	+	-
Eising et al. 2007	Experimental	*Sturnus vulgaris*	A4	Densidad reproductiva	+	0
Dentressangle et al. 2008	Experimental	*Sula nebouxii*	A4	Condiciones ambientales	-	+
Dentressangle et al. 2008	Experimental	*Sula nebouxii*	T	Condiciones ambientales	-	0
Dentressangle et al. 2008	Experimental	*Sula nebouxii*	A4	Atractivo del macho	-	+
Dentressangle et al. 2008	Experimental	*Sula nebouxii*	T	Atractivo del macho	-	0
Love et al. 2008	Correlativo	*Sturnus vulgaris*	CORT	Condición materna	-	+
Love et al. 2008	Correlativo	*Sturnus vulgaris*	CORT	Densidad reproductiva	+	-

Schmaltz et al. 2008	Correlativo	*Crotophaga ani*	T	Densidad reproductiva	+	0
Hargitai et al. 2009	Experimental	*Ficedula albicollis*	T	Invasión territorial/Agresión	+	+
Hargitai et al. 2009	Experimental	*Ficedula albicollis*	T	Densidad reproductiva	+	0
Guibert et al. 2010	Experimental	*Coturnix coturnix japónica*	T	Inestabilidad social	+	+
Safran et al. 2010	Correlativo	*Hirundo rustica erythrogaster*	T	Densidad reproductiva	+	-
Remeš 2011	Correlativo	*Parus major*	A4	Densidad reproductiva	+	+
Remeš 2011	Correlativo	*Parus major*	A4	Calidad del territorio	+	+
Remeš 2011	Correlativo	*Parus major*	T	Densidad reproductiva	+	0
Remeš 2011	Correlativo	*Parus major*	T	Calidad del territorio	+	0
Remeš 2011	Correlativo	*Parus major*	T	Atractivo del macho	+	+
Coslovsky et al. 2012	Experimental	*Parus major*	T	Riesgo de depredación	+	-
Coslovsky et al. 2012	Experimental	*Parus major*	A4	Riesgo de depredación	+	0
Paquet et al. 2013	Correlativo	*Philetairus socius*	T/A4/CORT	Ayudantes de crianza	+	-
Welty et al. 2012	Correlativo	*Athene cunicularia hypugaea*	T/A4/DHT	Densidad reproductiva	+	0
Almasi et al. 2012	Experimental	*Tyto alba*	CORT	Estrés materno	+	+
Bentz et al. 2013	Experimental	*Tachycineta bicolor*	T	Densidad reproductiva	+	+

Duckworth et al. 2015	Correlativo	*Sialia mexicana*	T	Competencia	+	+
Bentz et al. 2016b	Correlativo	*Sialia sialis*	T	Competencia	+	+
Lessells et al. 2016	Correlativo	*Parus major*	DHT/CORT	Temperatura ambiental	+	-
Lessells et al. 2016	Correlativo	*Parus major*	T/A4/E2	Temperatura ambiental	+	0
Schmaltz et al. 2016	Correlativo	*Crotophaga ani*	CORT	Densidad reproductiva	+	+
Hahn et al. 2017	Correlativo	*Setophaga ruticilla*	T	Parásitos de puesta	+	+
Hahn et al. 2017	Correlativo	*Catharus fuscescens*	T	Parásitos de puesta	+	0
Hahn et al. 2017	Correlativo	*Vireo olivaceus*	T	Parásitos de puesta	+	+
Hahn et al. 2017	Correlativo	*Molothrus ater*	T	Competencia	+	+
Langen et al. 2017	Experimental	*Coturnix japónica*	T/DHT/CORT	Densidad reproductiva	+	0
Bentz et al. 2018	Experimental	*Taeniopygia guttata*	T	Invasión territorial/Agresión	+	+
Miltiadous et al. 2019	Correlativo	*Erythrura gouldiae*	CORT	Condición materna	-	0
Mouton et al. 2022	Experimantal	*Sialia mexicana*	T	Riesgo de depredación	+	0

Impacto de las hormonas esteroides maternas durante el desarrollo temprano de las crías

Los efectos maternos han sido considerados como potencialmente adaptativos ya que podrían proveer información relevante sobre las condiciones ambientales a las que la descendencia podría enfrentarse después del nacimiento (Mousseau y Fox 1998, Berrigan y Scheiner 2004). Sin embargo, es posible que los beneficios en adecuación para la madre y la descendencia varíen dependiendo del contexto (Marshall y Uller 2007). Cuando las crías que fueron expuestas a hormonas maternas durante el desarrollo embrionario mitigan los efectos negativos de los ambientes hostiles similares a los que experimentó la madre en el momento de la puesta, y potencialmente incrementan su adecuación, se ha considerado que las hormonas depositadas en el huevo fueron el mecanismo que perfiló las características fenotípicas que confirieron las ventajas adaptativas (Groothuis et al. 2005). Trabajos en múltiples especies de aves han reportado que las hormonas maternas en el huevo tienen un impacto sobre la fisiología, conducta y rasgos de historia de vida de la descendencia. Por ejemplo, altos niveles de andrógenos en la yema pueden promover la eclosión más temprana de las crías al acelerar la tasa de crecimiento embrionario (Tabla 2), lo que podría acortar la duración del período de las crías en el nido, evitando el impacto de condiciones adversas propias de esta etapa, como parásitos o competencia entre hermanos en el nido, y favorecer la supervivencia en el corto plazo. De igual forma, la exposición a testosterona en la yema promueve mayor agresión, índices más altos de inmovilidad tónica y dominancia social, conductas que podrían favorecer la defensa de territorio, pareja y la adquisición de recursos (*e. g.* alimento o sitios de anidación) (Podmokła et al. 2018). Sin embargo, algunas de estas alteraciones al fenotipo podrían ser costosas para la progenie. Por ejemplo, altos niveles de andrógenos durante el período post-eclosión pueden comprometer la respuesta inmunitaria, aumentar los niveles de estrés oxidante (*i. e.* el desbalance entre las defensas antioxidantes y la producción de especies reactivas de oxígeno, moléculas altamente reactivas resultantes del metabolismo celular; Monaghan et al. 2009), y disminuir la probabilidad de supervivencia y reproducción de las crías, modificando su conducta y estrategias dispersivas (Tabla 2). Estos posibles costos infligidos por las hormonas maternas en el huevo han llevado a plantear la interrogante de si el embrión es un receptor pasivo de las hormonas maternas, y si una vez depositadas en el huevo, no existen mecanismos

complementarios que puedan afectar el grado de exposición del embrión, así como las consecuencias de esta exposición (Groothuis et al. 2020).

Acciones del embrión ante la exposición temprana a hormonas esteroides de origen materno
Recientemente, algunos estudios han mostrado que la concentración de hormonas en el huevo difiere a lo largo del período de incubación (Elf y Fivizzani 2002), lo que podría sugerir la participación activa del embrión regulando su exposición a altas concentraciones de hormonas maternas durante su desarrollo temprano (Gilbert et al. 2007). Se ha propuesto que los embriones en desarrollo podrían contar con mecanismos fisiológicos para metabolizar los andrógenos, estrógenos y glucocorticoides de origen materno (von Engelhardt et al. 2009, Paitz et al. 2011, Vassallo et al. 2014, Carter et al. 2018, Vassallo et al. 2019). Entre los mecanismos propuestos se encuentra la regulación a través de la acción de enzimas presentes en el embrión en desarrollo o en las membranas extra-embrionarias (*i. e.* tejidos accesorios que se forman a partir del embrión y que lo asisten en su desarrollo; Everaert et al. 2006) que podrían participar en el metabolismo de las hormonas durante el desarrollo embrionario. Por ejemplo, por medio de procesos enzimáticos como la sulfonación y la glucuronidación (mediante sulfotransferasas UDP-glucuronosiltransferasas, respectivamente) se producen metabolitos esteroideos fisiológicamente inactivos hidrosolubles que favorecen la difusión de la hormona hacia el albumen disminuyendo la exposición del embrión a ésta (Paitz y Bowden 2008, Paitz y Bowden 2009, Paitz y Bowden 2013). Se ha propuesto que, las enzimas aromatasa y 5α-reductasa pueden jugar un papel en la transformación de la testosterona en el huevo, ya que la actividad de estas enzimas convierte la testosterona a otras hormonas esteroides biológicamente activas (*i. e.* estradiol y 5α-dihidrotestosterona, respectivamente; Paitz y Bowden 2009, Campbell et al. 2020).

Tabla 2. Estudios experimentales que evalúan el efecto del incremento de la concentración de hormonas en el huevo (T: Testosterona, A4: Androstenediona, DHT: Dihidrotestosterona, CORT: Corticosterona, E2: Estradiol) en el fenotipo de la descendencia. +: Positivo, -: Negativo, 0: Sin diferencias.

Referencia	Especie	Hormona	Parámetro	Efecto en crías
Schwabl 1993	*Serinus canaria*	T	Dominancia/Agresión	+
Schwabl 1996	*Serinus canaria*	T	Crecimiento	+
Schwabl 1996	*Serinus canaria*	T	Solicitud de alimento	+
Sockman y Schwabl 2000	*Falco sparverius*	T+A4	Crecimiento	-
Sockman y Schwabl 2000	*Falco sparverius*	T	Supervivencia	-
Eising et al. 2001	*Larus ridibundus*	T+A4	Desarrollo embrionario	-
Eising y Groothuis 2003	*Larus ridibundus*	T+A4	Solicitud de alimento	+
Eising y Groothuis 2003	*Larus ridibundus*	T+A4	Supervivencia	+
Eising et al. 2003	*Larus ridibundus*	T+A4	Tasa metabólica	0
Andersson et al. 2004	*Coturnix chinensis*	T	Respuesta inmunitaria	+
Andersson et al. 2004	*Coturnix chinensis*	T	Crecimiento	0
Pilz et al. 2004	*Sturnus vulgaris*	T	Crecimiento	+
Pilz et al. 2004	*Sturnus vulgaris*	T	Solicitud de alimento	0
Pilz et al. 2004	*Sturnus vulgaris*	T	Supervivencia	+
Strasser y Schwabl 2004	*Passer domesticus*	T	Dominancia/Agresión	+
Strasser y Schwabl 2004	*Passer domesticus*	T	Ornamentos	+
Daisley et al. 2005	*Coturnix japonica*	T	Conducta intrépida	+
Tschirren et al. 2005	*Parus major*	T	Crecimiento	+
Ruboli et al. 2005	*Larus michahellis*	CORT	Desarrollo embrionario	+
Ruboli et al. 2005	*Larus michahellis*	CORT	Conducta intrépida	0
Ruboli et al. 2005	*Larus michahellis*	CORT	Respuesta inmunitaria	-/0
Groothuis et al. 2005	*Larus ridibundus*	T+A4	Respuesta inmunitaria	-
Müller et al. 2005	*Larus ridibundus*	T+A4	Respuesta inmunitaria	-
Eising et al. 2006	*Larus ridibundus*	T+A4	Dominancia/Agresión	+
Eising et al. 2006	*Larus ridibundus*	T+A4	Ornamentos	+
Janczak et al. 2006	*Gallus gallus domesticus*	CORT	Conducta intrépida	-
Janczak et al. 2006	*Gallus gallus domesticus*	CORT	Crecimiento	-

Navara et al. 2006a	*Haemorhous mexicanus*	T	Crecimiento	+
Navara et al. 2006a	*Haemorhous mexicanus*	T	Respuesta inmunitaria	+
Rubolini et al. 2007	*Phasianus colchicus*	T	Crecimiento	0
Rubolini et al. 2007	*Phasianus colchicus*	T	Ornamentos	-/0
Rubolini et al. 2006	*Larus chachinans*	T	Crecimiento	-
Müller et al. 2007	*Sturnus vulgaris*	T+A4	Supervivencia	+
Müller et al. 2007	*Sturnus vulgaris*	T+A4	Niveles hormonales circulantes	+
Tobler y Sandell 2007	*Taeniopygia guttata*	T	Conducta intrépida	+/-
Tobler et al. 2007	*Taeniopygia guttata*	T	Crecimiento	0
Tobler et al. 2007	*Taeniopygia guttata*	T	Tasa metabólica	+
Schwabl et al. 2007	Múltiples	DHT	Desarrollo embrionario	+
Saino et al. 2007	*Phasianus colchicus*	E2	Proporción de dígitos	-
Tschirren et al. 2007	*Parus major*	T	Estrategia dispersiva	+
Müller et al. 2008	*Serinus canaria*	T	Crecimiento	-
Müller et al. 2008	*Serinus canaria*	T	Dominancia/Agresión	0
Love y Williams 2008	*Sturnus vulgaris*	CORT	Supervivencia	-
Love y Williams 2008	*Sturnus vulgaris*	CORT	Crecimiento	-
Love y Williams 2008	*Sturnus vulgaris*	CORT	Solicitud de alimento	+
Love y Williams 2008	*Sturnus vulgaris*	CORT	Respuesta inmunitaria	-
Hegyi y Schwabl 2010	*Coturnix japonica*	DHT	Conducta intrépida	+
Hegyi y Schwabl 2010	*Coturnix japonica*	DHT	Crecimiento	0
Noguera et al. 2010	*Larus michahellis*	T	Daño oxidante	0
Noguera et al. 2010	*Larus michahellis*	T	Capacidad antioxidante	+
Nilson et al. 2011	*Taeniopygia guttata*	T	Tasa metabólica	+
Schwabl et al. 2012	*Passer domesticus*	T	Supervivencia	-
Ruuskanen et al. 2012	*Ficedula albicollis*	T+A4	Reclutamiento	-
Muriel et al. 2013	*Sturnus unicolor*	T/A4/T+A4	Desarrollo embrionario	+/-/0
Muriel et al. 2013	*Sturnus unicolor*	T/A4/T+A4	Crecimiento	-/+/'-
Muriel et al. 2013	*Sturnus unicolor*	T/A4/T+A4	Respuesta inmunitaria	Dependiente del sexo

Ahmed et al. 2014	*Gallus gallus domesticus*	CORT	Crecimiento	-
Ahmed et al. 2014	*Gallus gallus domesticus*	CORT	Dominancia/Agresión	+
Ahmed et al. 2014	*Gallus gallus domesticus*	CORT	Crecimiento	-
Ahmed et al. 2014	*Gallus gallus domesticus*	CORT	Niveles hormonales circulantes	+
Parolini et al. 2017	*Larus michahellis*	T	Estrés oxidante	-/+

Efectos maternos post-cigóticos: Acciones de la madre que afectan la exposición temprana a hormonas esteroides

Aunado a la potencial participación de la progenie en la exposición a hormonas durante el desarrollo embrionario es posible que, las variaciones en la concentración hormonal en la yema durante esta etapa, y consecuentemente los efectos en el fenotipo de la cría, respondan a alteraciones en los patrones de incubación. Por ejemplo, en algunas especies de reptiles se ha encontrado que la proporción sexual de la descendencia está influenciada por la temperatura de incubación posiblemente debido a su efecto sobre la activación de enzimas, como la aromatasa y 5α-reductasa las cuales compiten por el mismo sustrato hormonal, testosterona (Jeyasuria y Place 1998). En algunas especies aviares, por un lado, se ha encontrado que las hembras pueden modificar su conducta de incubación como consecuencia de cambios en los niveles de testosterona circulante en respuesta a factores ecológicos. Altas concentraciones de testosterona circulante en las hembras han sido vinculadas con menores tiempos de incubación, desarrollo más pobre del parche de incubación (*i. e.* menor área de superficie desprovista de plumas en la zona ventral del cuerpo de la hembra que permanece en contacto con los huevos durante la incubación) y, en consecuencia, temperaturas de incubación más bajas (Clotfelter et al. 2004, Rosvall 2013, Cantarero et al. 2015, de Jong et al. 2016). Estos resultados sugieren que, además de la influencia materna a través de la deposición de hormonas en el huevo, la conducta de incubación de las madres podría jugar un papel en la exposición a hormonas en el embrión. Por otro lado, estudios en individuos adultos han planteado que la testosterona podría alterar el fenotipo a través de su transformación a estrógenos por vía de la aromatización (Lynn et al. 2000, Quinn y Ottinger 2006, Casagrande et al. 2012). Sin embargo, este mecanismo ha sido poco estudiado en embriones en desarrollo. En conjunto, esta información sugeriría que las hormonas de origen materno podrían alterar el desarrollo de la progenie mediante su impacto directo o a través de su transformación a otro compuesto hormonal mediado por la conducta de incubación, la acción del embrión o la interacción del comportamiento de la madre y la participación del embrión. Una indagación integral sobre el destino de las hormonas de origen materno depositadas en el huevo y el rol del embrión ante la deposición de altas concentraciones de éstas permitiría determinar el mecanismo de acción de los efectos maternos mediados por hormonas. Adicionalmente, permitiría ampliar el conocimiento sobre los potenciales

mecanismos de ajuste y/o defensa con los que cuenta el embrión para lidiar con estas hormonas de origen materno, o determinar si éste es un receptor pasivo de las señales químicas de la hembra.

Hormonas esteroides circulantes en la descendencia

Si bien la mayor parte de los efectos maternos abordados en este trabajo se enfocan en los efectos maternos pre-cigóticos, mediados por hormonas a través de la deposición diferencial de éstas en la yema, el ambiente de los padres también puede influir en los niveles circulantes de hormonas en la descendencia sin necesidad de alterar el contenido hormonal del huevo. Una de las hormonas clave que puede estar influenciada por la exposición a eventos críticos predecibles e impredecibles en diferentes fases del ciclo de vida de los individuos es la corticosterona. El incremento en la concentración de corticosterona promueve un aumento en la ingesta de alimento y liberación de glucosa en sangre, así como la movilización y metabolismo de proteínas y lípidos (Sapolsky et al. 2000). Sin embargo, la exposición crónica (*e. g.* días o semanas) a corticosterona durante el desarrollo post-natal temprano induce cambios importantes en el fenotipo de las crías (Wingfield et al. 1998), como una reducción en la tasa de crecimiento y un incremento en los niveles de estrés oxidante (Wada y Breuner 2008, Müller et al. 2009, Costantini et al. 2011). El aumento en la concentración de corticosterona puede impactar el crecimiento indirectamente a través del aumento en los niveles de estrés oxidante, debido al rompimiento de lípidos y proteínas que además de energía, producen agentes pro-oxidantes (Taniguchi et al. 1999, Sapolsky et al. 2000, Spencer y Verhulst 2008, Stier et al. 2009, Schmidt et al. 2012). Por otro lado, un incremento en los niveles de corticosterona puede afectar directamente el crecimiento a través de la supresión de la proliferación celular (Orth et al. 1992). No obstante, no ha sido ampliamente investigado el efecto del ambiente pre-natal sobre los niveles hormonales circulantes en la progenie.

Objetivos generales

Esta tesis investigó si la competencia por cavidades de anidación experimentada por la madre modifica el ambiente hormonal (*i. e.* testosterona) de la descendencia durante el desarrollo embrionario y el período de crianza y, si consecuentemente, modifica rasgos conductuales, de

historia de vida y fisiológicos de las crías. Además, se evaluó si la exposición a testosterona de origen materno durante el desarrollo embrionario es modulada activamente por el embrión o a través del patrón de incubación de la madre o por la interacción de ambos factores transformando dicha hormona a otros metabolitos en la yema a lo largo el periodo de incubación. Para abordar estos objetivos generales, se usaron dos modelos de estudio: una población silvestre de azulejo de garganta azul (*Sialia mexicana*) que anida en cajas nido en el Parque Nacional La Malinche y huevos de codorniz japonesa (*Coturnix japonica*) obtenidos de ejemplares cautivos mantenidos en el Centro Tlaxcala de Biología de la Conducta (CTBC).

El azulejo de garganta azul es un modelo ideal para explorar el impacto de la competencia materna por el acceso a los sitios de anidación ya que al ser un usuario secundario de cavidades que depende fuertemente de la disponibilidad de cavidades para su reproducción y, al no contar con las adaptaciones morfológicas necesarias, no es capaz de excavar su propia cavidad, por lo que las cavidades son recursos limitados para ellas (Duckworth y Badyaev 2007, Burtka y Grindstaff 2015). El azulejo de garganta azul es un ave socialmente monógama que anida en Norteamérica y tiene un sistema de crianza cooperativa facultativa, donde algunos machos ayudan a sus padres en el siguiente evento reproductivo (Dickinson et al. 1996). Ambos sexos defienden el nido de conespecíficos y heterospecíficos (Dickinson et al. 1996, Duckworth 2006); y en particular, aquellas hembras que anidan en sitios con un número reducido de cavidades disponibles y, que compiten agresivamente por el acceso a ellas, depositan más testosterona en los huevos (Duckworth et al. 2015). Una mayor deposición de testosterona a los huevos se relaciona con la producción de crías macho que son más agresivas y que tienden a dispersarse del área natal durante la adultez (Duckworth y Badyaev 2007). Los machos no agresivos que se reproducen en el área natal y los machos agresivos que colonizan nuevos hábitats obtienen su valor máximo de adecuación cuando se reproducen en las poblaciones afines a su fenotipo (Duckworth 2008), es decir, existe una correlación fenotípica entre la conducta y las estrategias dispersivas mediada por la exposición hormonal durante el desarrollo temprano como resultado de la disponibilidad de recursos. Esto sugiere un valor adaptativo en la modificación de la cantidad de hormonas que las hembras depositan en el huevo (Duckworth 2008). Sin embargo, no se sabe si existen diferencias entre poblaciones en el efecto que tiene la competencia por recursos de anidación sobre la deposición de hormonas en huevo. Para evaluar

si la competencia por cavidades de anidación influye en la concentración de testosterona que las hembras de azulejo depositan en la yema del huevo, en el capítulo 1 se incrementó el nivel de competencia por medio de la manipulación experimental del número de cajas nido disponibles y la invasión simulada al territorio de un potencial competidor en un bosque con cavidades de reproducción limitadas. Se evaluó el efecto de la competencia materna por sitios de anidación sobre la concentración de testosterona en la yema, así como en la tasa de crecimiento, conducta exploratoria y probabilidad de supervivencia de las crías al emplumado (Figura 2). Específicamente, pusimos a prueba la hipótesis de que la competencia por cajas nido desencadena efectos maternos mediados por testosterona, esperando un incremento en la concentración de testosterona en la yema y que las crías expuestas a estas condiciones, tuvieran una menor probabilidad de eclosión, tasas de crecimiento post-eclosión más aceleradas, mayor frecuencia de conductas exploratorias y menor probabilidad de supervivencia al emplumado. De forma complementaria, en el capítulo 2 se exploró la influencia de la competencia materna por cavidades de anidación en la fisiología de la descendencia de crías de azulejo, por lo que se investigó si los niveles circulantes de corticosterona y de daño oxidante juegan un rol como un mecanismo que vincula la competencia femenina por sitios de anidación con la modificación en las trayectorias de crecimiento de la descendencia (Figura 2). Se esperaba que los niveles de corticosterona y daño oxidante en sangre fueran más altos y se relacionaran positivamente con la tasa de crecimiento de las crías de hembras experimentales en comparación con las crías de hembras controles.

En el capítulo 3, se probó experimentalmente la hipótesis de que la exposición a andrógenos maternos puede ser regulada a través de la temperatura de incubación mediante la acción de las enzimas presentes en el huevo, y la hipótesis de que el embrión participa activamente en el metabolismo de la testosterona. Con este propósito, se usó un sistema artificial de incubación y como modelo de estudio a la codorniz japonesa, ya que es una especie ampliamente utilizada en la investigación y los huevos pueden ser incubados exitosamente bajo condiciones de laboratorio (Figura 2). Se esperaba que, si la temperatura de incubación influye en la transformación de la testosterona, 1) los huevos con una mayor concentración de testosterona e incubados a 36° C tendrán niveles más altos de dihidrotestosterona y bajas concentraciones de estradiol y, por el contrario, 2) los huevos con altas concentraciones de

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testosterona e incubados a 38° C tendrán altos niveles de estradiol, pero bajos niveles de dihidrotestosterona. Pero, si la temperatura no influye en las hormonas en la yema, 3) se espera que huevos a los que se les incrementó experimentalmente los niveles de testosterona tendrán altas concentraciones de esta hormona independientemente de la temperatura de incubación y no se encontrarán diferencias en los niveles de dihidrotestosterona y estradiol. Asimismo, se espera que las predicciones 1) y 2) ocurrieran en huevos con un embrión en desarrollo, mientras que aquellos huevos donde el embrión no se desarrolló mostrarán el patrón de las predicciones 3).

Figura 2. Objetivos particulares planteados para evaluar el papel de las hormonas y la conducta en los efectos maternos pre y post-cigóticos en el azulejo de garganta azul (*Sialia mexicana*) y la codorniz japonesa (*Coturnix japonica*).

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CAPÍTULO 1

Competitive events during nest building influence offspring growth and behaviour, but not testosterone deposition in the egg yolk in Western Bluebirds (*Sialia mexicana*)**

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Running head: *Competition over breeding sites and offspring development*

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Competitive events during nest-building influence offspring growth and behaviour, but not testosterone deposition in the egg yolk in Western Bluebirds (*Sialia mexicana*)

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The environmental stressors that females face before egg-laying, such as competition for resources, can impact the fitness prospects of the female as well as her offspring through hormone-mediated maternal effects. In obligate secondary cavity-nesting species, suitable nest-holes are a limited resource, so gaining access to nest-sites may require competition with other individuals. In the Western Bluebird *Sialia mexicana*, we experimentally evaluated whether female exposure to increased competition for nest cavities (reduced available nest-holes plus exposure to a competitor's decoy and vocalizations) during the period of nest construction affects her offspring's developmental trajectory, survival probability and behaviour through an increase in the concentration of testosterone in the egg yolk. Chicks from experimental females had a lower body mass at age 3 days, followed by a faster growth rate during the rapid growth phase, a slower growth rate during the slow growth phase and larger tarsus at age 15 days (only male offspring). Additionally, experimental chicks exhibited a lower breathing rate. However, female exposure to stronger competition for nest cavities during nest construction did not affect testosterone concentration in the egg yolk. Differential testosterone deposition in yolk is therefore apparently not the underlying mechanism of the effects observed, which may be attributed to changes in females' parental behaviour triggered by competition for breeding sites.

Keywords: cavity-nesting species, maternal effects, *Sialia Mexicana*, yolk androgens.

The conditions under which an individual grows can influence its phenotype, and the environment experienced by the parents plays an important role in shaping those conditions (Mousseau & Fox 1998, Monaghan 2008). The influence that the mother's phenotype and/or the maternal environment can have on the offspring phenotype is defined as a 'maternal effect' (Uller 2008).

Maternal effects are considered to be potentially adaptive as they may provide relevant 'information' about environmental conditions likely to be experienced by the offspring (Mousseau & Fox 1998, Berrigan & Scheiner 2004, but see Marshall & Uller 2007).

Nest-site competition is one component of the environment of the mother that can induce maternal effects (Dhondt & Adriaensen 1999, Hernández-Brito et al. 2014, Fokkema et al. 2018). Nest cavities are a limited key resource for obligate secondary cavity-nesting species; individuals of these

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species cannot excavate their own holes, so they depend on cavities made by other birds or that naturally occur in dead trees (Guinan *et al.* 2008). In many cavity-nesting species, females aggressively defend nest-sites from intruders (Gowaty & Wagner 1988, Sandell & Smith 1997, Rosvall 2008) and the intensity of these competitive interactions has been shown to influence the phenotype of the offspring (Mazuc *et al.* 2003, Duckworth *et al.* 2015). The mechanisms underlying the action of those maternal effects can range from variations in parental behaviour (Jacot *et al.* 2009, Siefferman & Burdick 2020, Song *et al.* 2016) to direct transfer of substances that may affect embryo development, such as micronutrients (Biard *et al.* 2009, Mentesana *et al.* 2019), antibodies (Buechler *et al.* 2002, Gasparini *et al.* 2007, Tschirren *et al.* 2009) and hormones (East *et al.* 2009, Bentz *et al.* 2013, Crespi & Warne 2013, Hsu *et al.* 2017, Ruuskanen & Hsu 2018).

The transfer of maternal testosterone into the egg yolk has been one of the most frequent targets of investigation into hormone-mediated maternal effects promoted by female competition over breeding sites, yet the fitness effects of testosterone-mediated maternal effects are still debatable (Podmokła *et al.* 2018). Numerous studies have shown that an increased concentration of testosterone in the egg yolk may have positive consequences on the development (Muriel *et al.* 2013, but see Smiseth *et al.* 2011) and behaviour (Ruuskanen *et al.* 2018) of the offspring. However, increased levels of testosterone in the egg may also entail costs such as increased oxidative damage to biomolecules (Parolini *et al.* 2019), a decline in the immune response (Navara *et al.* 2005), and reduced hatching and fledgling probability (Schwabl *et al.* 2012). Furthermore, inconsistent results regarding maternal effects triggered by increased competition among species or populations from the same species have been reported (e.g. Navara *et al.* 2006, Bentz *et al.* 2016a, Albers *et al.* 2017), suggesting that differences in life histories and ecological conditions among populations may influence the occurrence of testosterone-mediated maternal effects as a response to increased competition over key resources.

Western Bluebirds *Sialia mexicana* are socially monogamous passerines that breed from the South of Canada to Central Mexico. This species depends on cavity availability for reproduction,

and cavities are a limited resource (Holt & Martin 1997, Duckworth 2006a, Burtka & Grindstaff 2015). During a breeding attempt, reproductive males usually obtain the territory, females build the nest and both sexes defend the cavity against intruders (Dickinson *et al.* 1996, Duckworth 2006b). Western Bluebirds aggressively compete with other secondary cavity-nesters, and in northern populations, these competitive interactions during early stages of egg formation trigger a testosterone-mediated maternal effect on the dispersive and aggressive phenotype of offspring at sexual maturity (Duckworth *et al.* 2015). Females exposed to a low density of nest cavities or an increased number of territorial intrusions deposit higher levels of testosterone in the eggs and, consequently, male offspring exhibit an aggressive and dispersive phenotype during adulthood (Duckworth *et al.* 2015). However, competitive conditions for nest-sites vary through the range of distribution of Western Bluebirds, and this variation may influence female breeding strategies, the stimulus that triggers maternal effects and the type of maternal effects displayed. Compared with northern migrant populations, Western Bluebirds from populations of Central Mexico do not migrate latitudinally (Guinan *et al.* 2008), and exhibit a shorter breeding season (April–June vs. April–August in Montana), with only one breeding attempt successfully completed per reproductive season (Potticary & Duckworth 2018, 2020, Porras-Reyes *et al.* 2021). These ecological differences could influence the female's response towards competitive events and, therefore, the maternal effects on offspring phenotype that it could trigger. A feasible scenario that deserves attention is that, in a resident population, the competition over breeding sites could be more intense or prolonged, i.e. all year-round, as has been reported in other territorial passerines (Gill *et al.* 2007), and the potential consequences of these differences on maternal effects deserves further attention. Moreover, in particular, in *Sialia mexicana*, the influence of nesting-site limitation on offspring phenotype through maternal effects has been investigated during the offspring's adulthood (Duckworth *et al.* 2015), yet earlier influences have received less attention.

In the present study, using a southern population of Western Bluebirds, we experimentally increased the level of competition for nest cavities during the nest-building period by decreasing the

number of available nestboxes and simulating intrusions of potential competitors. We hypothesized that if there is a testosterone-mediated maternal effect trigged by increased competition over nestboxes, as reported in northern populations of the same species, augmented competition over nest cavities would lead to: (1) increased concentration of testosterone in the egg yolk and, as a result of this maternal effect, (2) lower hatching success, due to possible higher embryo mortality, and (3) nestlings with higher growth rates, as testosterone stimulates growth factors and cell proliferation, and higher scores in two proxies of exploratory behaviour and risk-taking phenotypes (breathing rate and tonic immobility), but lower fledging probability.

METHODS

Experimental procedures

This study was conducted in La Malinche National Park, Tlaxcala, Mexico (19°14′N, 98°02′W) on a population of Western Bluebirds that has been breeding in nestboxes installed from 2009 onwards (Cuatianquiz-Lima & Macias-García 2016). We experimentally manipulated females' competition for nest cavities by reducing the number of nestboxes available near an occupied nest (hereafter, focal nest) and increasing the perceived presence of competitors through intrusion trials (see below) during the nest-building stage, as in this period Bluebirds are susceptible to evictions and the period coincides with the time of yolk deposition during the process of egg formation (Badyaev et al. 2005, Navara et al. 2006). Previous to the start of the reproductive season, during February and March, we installed an additional nestbox 5–10 m apart from each focal nestbox that was successfully occupied for Bluebirds in the reproductive seasons of 2013–2018. Hence, the nearest nest to each focal occupied nestbox (before any control or experimental treatment was performed) was 5–10 m apart and, when it was not removed due to the experimental manipulation (see below), it was never occupied by a different reproductive pair. Nestboxes were roughly evenly distributed within the study area and adjacent boxes were on average 60 m apart (range: 22–125 m). From April to May, all boxes were visited twice a week; once nesting material (grasses and pine needles) was observed inside the nestbox, nests were alternately

assigned to either the control or experimental treatment. In the experimental group, when nest-building was first recorded in a given nest, the nearest nestbox (installed at a distance of 5–10 m) was removed to change the female's perception of the availability of cavities in her territory. In this group, intrusion trials consisted of the presentation of a 3D model and a playback with vocalizations of a heterospecific, the House Wren *Troglodytes aedon*. In this study population, the House Wren is one of the main competitors of Western Bluebirds for nest cavities. Displacement of Western Bluebirds from a cavity is often observed after successive events of superposition of nesting material between these two species, with the House Wren having a higher probability of taking over the nest cavity, yet no evidence has been found of House Wrens destroying Bluebird eggs or killing nestlings (B. Montoya pers. obs.). In the control group, no boxes were removed so the control females had at least one empty box within their territory. Control intrusion trials were performed following the same protocol described above for the experimental group, but the decoy and playbacks were of a Red Warbler *Cardellina rubra*. Red Warblers breed in the same area as Western Bluebirds but build their nests on the ground and therefore do not compete for nest cavities. The number of nestboxes (with nest-building activity recorded) within the territory of each active nest included in the study (radius of 150 m) did not differ between treatments (linear model (LM) with normal error distribution: $F_{1,48} = 0.05$, $P = 0.82$).

During each intrusion trial (either experimental or control), a speaker was placed at the side of the focal nestbox hung on a stick, with the 3D model perched on the top of the speaker. Decoys of species used in the intrusion trials were modelled with coloured ethylene-vinyl acetate rubber foam. We did not use a conspecific for territorial intrusion to avoid the possible occurrence of ovicide and pair separation (Duckworth 2006a). The playback of the intrusion trial started when the female of a focal nest was visually identified (during all the trails the females were outside the box), and was played for 10 min (either experimental or control). No playback was reproduced when the female of the focal nest was not sighted for 30 min after the placement of the decoy by the side of the nestbox. Recordings of House Wren and Red Warbler vocalizations were obtained from a virtual song library (http://xeno-canto.org. Catalogue No.:

XC302620 and XC71076) and played using a wireless speaker (Link bits Bluetooth speaker, frequency response: 100 Hz to 20 kHz, signal to noise ratio ≥ 80 dB). Intrusion trials, in the experimental and control groups, were performed twice a week, between 08:00 and 15:00 h (the peak period of activity of Bluebirds), until clutch completion was recorded. The time elapsed between the last territorial intrusion and the laying of the egg was 2–5 days (range: 0–11 days before first egg-laying); this timing is similar to previous studies (on average 3 days before egg-laying; Mazuc et al. 2003, Navara et al. 2006, Bentz et al. 2013). Considering that Bluebirds defend their territories throughout the whole breeding period (Gowaty 1981) and our aim was to manipulate conditions exclusively during the nest-building period, the box closest to each control nest (within 5–10 m) was removed once the clutch was completed. This procedure aimed to maintain similar conditions between the control and experimental nests during the period of incubation and post-hatching parental care. During the intrusion trails, experimental females were more prone to defend their nestbox than were control females (latency from decoy presentation to the arrival of the female at the nestbox, a generalized linear mixed model (GLMM) with normal error distribution: experimental = 14.04 ± 1.65 min, control = 23.12 ± 1.66 min; treatment × laying date: $\beta = 0.95 \pm 0.30$; $F_{1,19.71} = 10.03$, $P = 0.005$).

Fifty nestboxes were included in the study (26 experimental and 24 control); 10 pairs (five pairs in each group) stopped nest-building and deserted the nest before laying any eggs. Control and experimental nests did not differ in the number of intrusion trials received (generalized linear model (GLM) with Poisson error distribution (log link function): experimental = 3.96 ± 2.24, control = 4.32 ± 2.55; $z = 0.70$, df = 187, $P = 0.486$).

All nests were visited every 3 days to track nest-building activity and laying by Western Bluebirds or any other species. During the incubation and nestling periods, nests were visited every 3 days until chicks fledged to record the loss of any egg or chick. Egg length and width were measured with a calliper (± 0.02 mm) and a yolk biopsy was taken from one recently laid egg (2.58 ± 0.25 days after laying) from each clutch (see below). Individual egg volume was calculated as $0.51 * length * width^2$ (Hoyt 1979). At ages 3, 9 and 15 ± 1 days post-hatching, we measured

nestlings' tarsus and ulna length using callipers (± 0.02 mm) and body mass with a digital balance (± 0.01 g). From hatching to 15 days, chicks were individually identified with non-toxic and water-resistant nail polish on the toenails, and fledglings were ringed with coloured and numbered poly-methylmethacrylate leg bands (Interrex, Poland). At age 3 days, 50 µL of blood was taken from the jugular vein for molecular sexing (Fridolfsson & Ellegren 1999).

In 33 of the 50 nests initially included in the experiment, at least one egg was laid ($n = 20$ experimental and 13 control). Nests with only one egg laid were always deserted by the breeding pair, so they were not included in the analyses ($n = 3$), and three additional clutches were predated. Seventy-seven chicks hatched from 25 nestboxes, and 60 chicks from 22 nestboxes survived to age 15 days.

Yolk biopsy

In Western Bluebirds, intra-clutch variation in the concentration of testosterone levels in the yolk is low (≈ 35%, Duckworth et al. 2015). Therefore, to reduce the potential impact of yolk biopsy on the success of the reproductive attempt of the pair, we randomly selected one egg per clutch and a yolk biopsy was performed between zero and two days after clutch completion following the protocol of Duckworth et al. (2015). Briefly, eggs were placed with the short end up, lighted with a LED lamp to visualize the yolk, a small area of the shell was sterilized with 95% ethanol, and the needle (31G × 6 mm) of a sterile insulin syringe (0.3 mL) was inserted into the egg at a 45° angle to the long axis of the egg to obtain 40 µL of yolk. Samples were stored in 100 µL deionized water, maintained on ice during transportation to laboratory facilities, and stored at −80°C until analyses. The shell was sealed with surgical glue to decrease the risk of infection. Twenty-six eggs (16 experimental and 10 control) were sampled. We did not collect yolk samples from seven nests because two nests were predated before clutch completion, three nests were abandoned by females after laying the first egg, and, in the remaining two, embryo development was advanced by the time of yolk sampling. No association was found between the concentration of yolk testosterone and the number of days the egg was incubated before the biopsy was performed (range 910.77–17 384.38 pg/mL;

LM with normal error distribution: $F_{1,24} = 0.24$, $P = 0.627$). Yolk collection took less than 5 min. However, egg biopsy decreased the hatching success of the sampled eggs; 65 of 70 (92.86%) non-biopsied eggs hatched, whereas 12 of 26 (46.15%) sampled eggs hatched. Nestlings from biopsied eggs were included in the subsequent analyses, except for hatching success.

Behavioural records

To assess whether female competition for a nest-box affected Western Bluebird offspring exploratory behaviour, at ages 15 and 17 days all chicks in the study were tested for tonic immobility and breathing rate (Fuciková *et al.* 2009, Ruuskanen & Laaksonen 2010). Previous studies have found a positive relationship between breathing rate (estimated as the number of breast movements per unit of time) and exploratory behaviour in 14-day-old Great Tit *Parus major* nestlings (Fuciková *et al.* 2009). The time taken to recover upright position after tonic immobility (i.e. when the bird is placed on a surface laying on its back) has been found to be inversely related to novel environment exploration by chicks, and hence has been considered to be a proxy of the individual's boldness (Mills & Faure 1991, Ruuskanen & Laaksonen 2010). For both tests, nestlings were held laid on their backs on the palm of an open hand and the breathing rate (recorded as the time required for the chick to complete 20 breast movements) and the tonic immobility (the time required to recover upwards position) were quantified. Breathing rate and tonic immobility were quantified always by the same observer (A.H.).

Testosterone quantification

To estimate the concentration of testosterone in the biopsied egg yolks, we used an enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Montgomery, PA, USA). Steroid hormones in the yolk samples were extracted by adding 1 mL diethyl ether and allowing the sample to evaporate. This procedure was repeated twice; 1 mL methanol was then added and left to evaporate (López-García *et al.* 2013). Extracts were re-suspended by adding 150 μL ultrapure water, and 10 μL of this re-suspension was added to 290 μL ELISA buffer. Hormone quantification was performed following the manufacturer's instructions.

Each sample was quantified in duplicate and absorbance values were interpolated into a reference curve of testosterone. Results are expressed as pg testosterone/mL. Intra-plate variation was 1.70% and inter-plate variation 1.87%.

Statistical analyses

All analyses were conducted in R version 3. 5. 2 (R Development Core Team 2018). Final models were obtained by stepwise backward deletion of non-significant terms ($P > 0.05$). The sample sizes differ slightly among analyses due to nestling mortality or missing data. The presence of helpers at the nest was not considered in the analyses due to the low occurrence; there were helpers at four experimental nests and no control nests. The influence of nestling sex and its interaction with the treatment on offspring growth, behaviour and survival was analysed in independent models but no effect was identified ($P > 0.05$). Therefore, to avoid model overparameterization, those variables were excluded from the analyses (but see below). However, because availability of nestboxes can decrease as breeding season progresses, perceived competition due to the experimental manipulation may be linked to the specific laying/hatching date of the studied nest. Therefore, we included laying or hatching date as covariates in the statistical models. The experimental manipulation had no effect on laying probability, laying date, laying latency, individual egg volume or clutch size (Supporting Information Appendix S1).

Hatching probability was analysed by fitting a GLMM with binomial error distribution and *logit* link function (package *lme4*; Bates *et al.* 2015), with the nest identity as a random factor, treatment as a fixed factor, laying date and individual egg volume (to control for a bias in maternal initial investment) as covariates, and the interactions of the treatment with individual egg volume and laying date. Eggs sampled for testosterone determination were excluded from this analysis.

Prior to statistical analyses, testosterone concentrations were log-transformed to meet normality assumptions. To evaluate the effect of the treatment on yolk testosterone levels, we fit an LM including laying order and the interaction between the treatment and laying order. Laying order was included in the model as a continuous variable due to the lack of statistical power to include a four-level factor and test for its interaction with

the experimental treatment (i.e. Schielzeth 2010). Even though intra-clutch variation in yolk testosterone levels reported for *Sialia* spp. is thought to be low (Navara *et al.* 2006, Duckworth *et al.* 2015), we included laying order in the analyses because variation in hormone content among eggs within a clutch might still be biologically meaningful.

We also analysed the effect of the treatment on nestling growth by fitting GLMMs with a normal error distribution, including in each model the nest identity as a random effect to control for the non-independency of chicks belonging to the same nest. Body mass, and tarsus and ulna length at ages 3 and 15 days were analysed, including treatment as a fixed effect, hatching date as a covariate and the interaction of the treatment with hatching date. Additionally, in models analysing tarsus length at age 15 days, chick sex and its interaction with the treatment were included. To analyse the effect of the treatment on body size and mass gain, we divided the nestlings' development period into two phases – rapid and slow growth. The rapid growth phase was defined as the period from 3 to 9 days post-hatching, during which chicks grow exponentially, which is followed by the slow growth phase from age 9 to 15 days, during which the growth rate is slower and reaches a *plateau* (Montoya *et al.* 2020). The gain in body mass, tarsus and ulna length was computed as follows: *(measurement at final age – measurement at initial age)/measurement at initial age*. Models included treatment as a fixed factor, hatch date as a covariate and the interaction of the treatment with hatching date. Independent models to analyse each morphological trait were fit because the treatment may have had a tissue-specific effect or promote a developmental hierarchy of body structures over time, similar to results reported after periods of food shortage in nestlings from multiple species (Bize *et al.* 2006, Hegyi & Török 2007, Cleasby *et al.* 2011).

The effect of treatment on the breathing rate and tonic immobility was evaluated using independent repeated-measures GLMMs with normal error distribution. Models included the treatment as a fixed factor, hatching date as a covariate, the interaction between treatment and hatching date, and chick identity nested in nest identity as a random factor.

The effect of the experimental manipulation on offspring survival at age 15 days was analysed by fitting a GLMM with binomial error distribution and a logit link function. The treatment, hatching date and interaction between treatment and hatching date were included as fixed effects, and the nest identity as a random factor.

Finally, the effect size for principal results was calculated as ϵ^2_p for GLMMs with normal error distribution and as odds ratio for GLMMs with binomial error distribution, using the package *effectsize* (Ben-Shachar *et al.* 2020).

RESULTS

Hatching probability was unrelated to the treatment ($z = 1.43$, df = 67, $P = 0.152$), laying date ($z = 0.06$, df = 66, $P = 0.953$) or egg volume ($z = -0.01$, df = 65, $P = 0.994$). Moreover, the interactions between treatment and laying date ($z = -1.21$, df = 64, $P = 0.228$) or treatment and egg volume ($z = -1.23$, df = 63, $P = 0.219$) did not influence hatching probability.

Testosterone concentration in the egg yolk was not associated with the experimental manipulation (experimental group: 4426.84 ± 1108.84 pg/mL; control group: 6201.16 ± 1294.76 pg/mL; $F_{1,24} = 2.833$, $P = 0.105$, Fig. 1), laying order ($F_{1,23} = 0.387$, $P = 0.540$), or the interaction between experimental manipulation and laying order ($F_{1,22} = 0.191$, $P = 0.666$; Supporting Information Fig. S1).

Chicks from the experimental group had a lower body mass at age 3 days when accounting for hatching date in the analysis (Table 1, Fig. 2a);
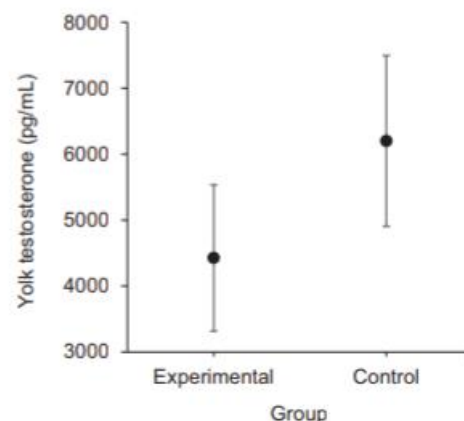

Figure 1. Yolk testosterone concentration of eggs laid by experimental and control females: mean ± se.

similar conclusions were reached when nestlings with extremely late hatching date were excluded from this analysis ($\beta = -0.21 \pm 0.10$, $F_{1,20.53} = 4.17$, $P = 0.054$, $\varepsilon^2_p = 0.13$). Experimental chicks that hatched later in the season gained more body mass during the rapid growth phase compared with control and early hatched experimental chicks (Table 1, Fig. 2b). Tarsus length at age 3 days was negatively related to hatching date (Table 1). However, tarsus and ulna length did not differ between experimental and control nestlings at age 3 days or during the rapid growth phase (Table 1). In contrast, during the slow growth phase (ages 9–15 days), experimental nestlings gained less ulna length (Table 2, Fig. 2c). Body mass and tarsus length were unrelated to treatment, hatching date or their interaction when tested during the slow growth phase (Table 2). At age 15 days, experimental male nestlings (but not females) had larger tarsus lengths (Table 2, Fig. 2 d). Experimental manipulation, hatching date and the interaction of treatment with hatching date were all unrelated to nestling body mass and ulna length at age 15 days (Table 2).

Experimental nestlings exhibited a slower breathing rate (Table 3, Fig. 3). All other variables analysed were unrelated to the offspring's breathing rate (Table 3). Offspring behaviour in the tonic immobility test was unrelated to the treatment, hatching date, or interaction between treatment and hatching date (Table 3).

We found that fledging survival probability tended to be lower for offspring in the experimental group than in the control group, although it was not statistically significant ($\beta = -3.76 \pm 2.05$, $z = -1.84$, df $= 75$, $P = 0.066$, odds ratio $= 43.06$). Sixteen of 49 (33%) experimental chicks died vs. one of 28 (3%) control nestlings. Fledgling survival was unrelated to hatching date ($z = -0.85$, df $= 73$, $P = 0.396$) or the interaction between the treatment and hatching date ($z = -0.61$, df $= 72$, $P = 0.543$).

DISCUSSION

Our results suggest that, in Western Bluebirds, experimentally increasing competition during the nest-building period might affect the offspring's growth trajectory and breathing rate. Thus, although experimental chicks were slightly smaller initially, they exhibited what could be considered compensatory growth during the rapid growth period, to reach the same overall size (or, in the case of males, slightly larger tarsus size) as chicks from the control group by the end of the nestling period. Moreover, experimental nestlings appeared to have a slower breathing rate than control chicks. The effects of the experimental manipulation

Table 1. Initial body size and body size gain of offspring from experimental and control nests during the rapid growth phase.

	Initial body size				Body size gain during rapid growth phase			
	Estimate $\pm$ se	$F(\varepsilon^2_p)$	df	P	Estimate $\pm$ se	$F(\varepsilon^2_p)$	df	P
Body mass								
Treatment	27.40 ± 13.40	4.18	1, 21.89	0.053	-11.37 ± 5.47	4.32	1, 18.63	0.052
Hatching date	0.11 ± 0.08	0.07	1, 21.92	0.787	-0.03 ± 0.03	0.43	1, 18.61	0.522
Treatment $\times$ Hatching date	**-0.19 ± 0.09**	**4.22 (0.12)**	**1, 21.92**	**0.052**	**0.08 ± 0.04**	**4.54 (0.15)**	**1, 18.61**	**0.047**
Tarsus length								
Treatment	-0.12 ± 0.50	0.06	1, 21.98	0.812	0.11 ± 0.08	2.28	1, 19.24	0.148
Hatching date	**-0.05 ± 0.03**	**4.30 (0.13)**	**1, 22.00**	**0.050**	0.01 ± 0.08	2.00	1, 19.13	0.173
Treatment $\times$ Hatching date	-0.11 ± 0.06	3.01	1, 21.44	0.097	0.02 ± 0.01	2.54	1, 16.92	0.129
Ulna length								
Treatment	0.50 ± 0.42	1.37	1, 23.36	0.254	0.06 ± 0.13	0.25	1, 19.74	0.622
Hatching date	-0.03 ± 0.02	1.39	1, 21.58	0.251	0.01 ± 0.01	0.51	1, 20.61	0.483
Treatment $\times$ Hatching date	-0.08 ± 0.05	2.16	1, 22.67	0.155	0.02 ± 0.02	0.91	1, 18.73	0.353

Note: In the experimental group, females bred in territories where the number of nest cavities was reduced by removing the nearest nestbox and were exposed to simulated intrusions by a competitor. In the control group, females bred in territories where no boxes were removed, and intrusion trials were conducted using a non-competitor. Sample sizes for each analysis were distributed as follows: experimental group age 3 days ($n = 47$) and rapid growth phase ($n = 37$); control group age 3 days ($n = 28$) and rapid growth phase ($n = 28$). F- and P-values in bold indicate variables included in the final model after excluding non-significant terms.

Figure 2. Body growth and size differences between experimental and control chicks: (a) body mass at age 3 days, considering hatching date; (b) body mass gain during the rapid growth phase (age 3–9 days), accounting for hatching date; (c) ulna length gain (mean ± se) during the slow growth phase (age 9–15 days); and (d) tarsus length (mean ± se) at age 15 days in male and female offspring. Note that results in panel (a) remain qualitatively the same when the three hatchlings with the latest hatching date (all from the same nest) are removed from the analysis ($F_{1, 20.53} = 4.17$, $P = 0.054$).

occurred without a concurrent increase in the concentration of yolk testosterone in eggs laid by experimental females. These results may suggest that competition over nest-sites during the nest-building stage influences offspring phenotype. However, it seems unlikely that, in the population of Western Bluebirds studied here, these phenotypic changes are due to differential deposition of testosterone in eggs.

Contrary to prediction, no differences in testosterone deposition in egg yolk were found in response to increased competition for nest-sites. This lack of difference between treatment groups might arise if testosterone concentration varies with laying order and with the time elapsed since egg-laying because, in our experiment, testosterone was estimated from a randomly selected egg from each nest, regardless of its laying order, resulting in variation in the number of days between laying and biopsy. However, this is unlikely because concentration of yolk testosterone and laying order were unrelated. Another potential explanation for the lack of difference between treatment groups in testosterone deposition in egg yolks is that the experimental manipulation was insufficient to increase the level of competition perceived by females, and hence the maternal testosterone deposited in the eggs. However, the experimental manipulation used here, i.e. simulated intrusions and reduction of available nest-sites, has been found to increase perceived competition in several passerine species, including Western Bluebirds, resulting in an increase in females' defensive and aggressive behaviours and testosterone deposition in the eggs (Whittingham & Schwabl 2002, Mazuc et al. 2003, Pilz & Smith 2004, Navara et al. 2006, Bentz et al. 2013, Duckworth et al. 2015). In our study, females were exposed to the experimental

Table 2. Final body size and body size gain of offspring from experimental and control groups, during the slow growth phase.

	Body size gain during slow growth phase				Final body size			
	Estimate $\pm$ se	$F(\epsilon^2_p)$	df	P	Estimate $\pm$ se	$F(\epsilon^2_p)$	df	P
Body mass								
Treatment	-0.17 ± 0.09	3.40	1, 18.52	0.081	1.06 ± 0.96	1.21	1, 15.95	0.288
Hatching date	-0.002 ± 0.007	0.10	1, 16.87	0.757	0.05 ± 0.07	0.44	1, 13.41	0.519
Treatment × Hatching date	-0.0005 ± 0.01	0.001	1, 15.98	0.974	-0.12 ± 0.15	0.71	1, 13.31	0.415
Tarsus length								
Treatment	-0.04 ± 0.05	0.79	1, 20.72	0.383	0.31 ± 0.64	3.34	1, 18.47	0.081
Hatching date	-0.0004 ± 0.004	0.01	1, 18.08	0.917	0.03 ± 0.05	0.48	1, 17.30	0.498
Nestling sex	–	–	–	–	-1.08 ± 0.31	1.02	1, 32.63	0.319
Treatment × Nestling sex	–	–	–	–	**1.71 $\pm$ 0.45**	**14.20 (0.28)**	**1, 32.74**	**< 0.001**
Treatment × Hatching date	0.001 ± 0.007	0.02	1, 17.17	0.880	-0.05 ± 0.10	0.56	1, 16.36	0.464
Ulna length								
Treatment	-0.14 ± 0.06	**6.24 (0.21)**	**1, 18.42**	**0.022**	1.15 ± 0.91	1.59	1, 17.25	0.223
Hatching date	-0.004 ± 0.005	0.79	1, 16.24	0.353	-0.04 ± 0.07	0.26	1, 15.75	0.616
Treatment × Hatching date	0.004 ± 0.01	0.25	1, 15.01	0.623	0.02 ± 0.14	0.01	1, 15.02	0.915

Note: In the experimental group, females bred in territories where the number of nest cavities was reduced by removing the nearest nestbox and were exposed to simulated intrusions by a competitor. In the control group, females bred in territories where no boxes were removed, and intrusion trials were conducted using a non-competitor. Sample sizes for each analysis were distributed as follows: experimental slow growth phase ($n = 29$) and age 15 days ($n = 29$, females = 22, males = 7); control group slow growth phase ($n = 28$) and 15 days ($n = 25$, females = 14, males = 11). F- and P-values in bold indicate variables included in the final model after excluding non-significant terms.

Table 3. Experimental treatment effect on tonic immobility and breathing rate of nestlings.

	Tonic immobility				Breathing rate			
	Estimate $\pm$ se	F	df	P	Estimate $\pm$ se	$F(\epsilon^2_p)$	df	P
Treatment	2.20 ± 5.54	0.16	1, 56.24	0.692	**0.54 $\pm$ 0.27**	**3.92 (0.12)**	**1, 20**	**0.050**
Hatching date	0.07 ± 0.81	0.004	1, 53.44	0.952	0.0001 ± 0.02	0.001	1, 11.63	0.994
Treatment × Hatching date	0.02 ± 0.40	0.68	1, 52.44	0.414	0.03 ± 0.04	0.58	1, 11.53	0.525

Note: In the experimental group, females bred in territories where the number of nest cavities was reduced by removing the nearest nestbox and were exposed to simulated intrusions by a competitor. In the control group, females bred in territories where no boxes were removed, and intrusion trials were conducted using a non-competitor. Experimental group $n = 34$, control group $n = 25$. F- and P-values in bold indicate variables included in the final model after excluding non-significant terms.

manipulation during the period of egg formation (Badyaev *et al.* 2005, Navara *et al.* 2006) and, overall, we used a similar procedure as in previous studies (Mazuc *et al.* 2003, Navara *et al.* 2006, Bentz *et al.* 2013). Hence, it seems likely that females in the experimental group of our study did experience augmented apparent competition. The fact that our results suggest the occurrence of treatment effects on nestling growth and breathing rate (see below) supports, to some extent, the idea that the manipulation did affect females.

An alternative explanation for the absence of treatment effects on testosterone deposition in the egg is that females in our study differ from females of northern populations in their response to increased competition, for example by modifying egg components other than testosterone (e.g. other hormones, micronutrients) and/or pre- and post-hatching parental behaviour. Accordingly, in the presence of higher densities of a competitor (Tree Swallows *Tachycineta bicolor*), Eastern Bluebird *Sialia sialis* parents increase the coordination of
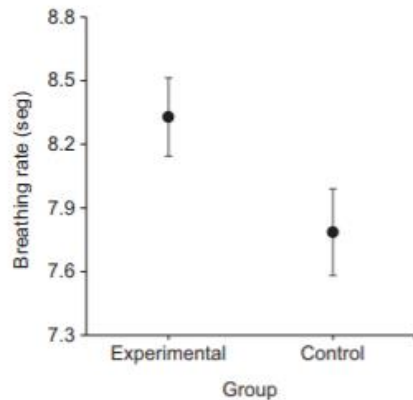
Figure 3. Breathing rate (time to complete 20 breast movements) of nestlings from the experimental and control group: mean ± se.

their feeding visits to offspring, promoting a faster nestling development (Siefferman & Burdick 2020). Differences between populations may be linked to the fact that habitat conditions vary between study sites. For example, in La Malinche National Park, during the breeding season, temperature ranges from −1 to 23 °C (Porras-Reyes et al. 2021), whereas in the northern populations, temperature ranges from −12 °C, with rain or snowstorms, to 22 °C. Low temperatures in northern populations have been shown to produce a rise in female corticosterone levels, influencing reproductive performance and favouring the production of aggressive male offspring (similar to the effect of increased competition for nest-sites, Potticary & Duckworth 2020). In keeping with the idea of ecological differences, the amount of naturally occurring competition experienced by the population studied here may reach levels experienced by experimental groups from other populations studied, so that our experimental manipulation 'saturated' the females' capacity to respond. Hence, the ecological factors that may influence variation in the responses to exhibit a similar stimulus among populations of the same species deserve further study.

Interestingly, although testosterone did not increase in the eggs of experimental females as we expected, the increased competition during the nest-building period did influence the growth trajectories of offspring and promoted the growth of larger tarsal bones among male fledglings,

suggesting that these effects could be mediated by other mechanisms besides the previously reported effect of egg yolk testosterone. Experimental nestlings that hatched later in the season were slightly lighter at the age of 3 days but gained more body mass during the rapid growth phase than control nestlings. These differences in growth between control and experimental nestlings were probably not associated with pre-hatching effects of the treatment, as other pre-hatching traits such as laying date, laying latency and clutch size (see Supporting Information Appendix S1), as well as yolk testosterone were not affected by the treatment. Moreover, during the slow growth phase, chicks from the experimental group showed lower ulna growth rates compared with control nestlings. The overall results thus might suggest that the treatment may have affected offspring growth through post-hatching effects, such as variation in parental behaviour, similar to what has been reported in Eastern Bluebirds (Siefferman & Burdick 2020). Sex-specific differences in tarsus length were found at age 15 days, near the time of fledging, in which males from the experimental group had larger tarsi than control males. Although we do not know the mechanism underlying these differences in the growth trajectories of experimental nestlings in the population studied, this result coincides with reports in northern populations, where Western Bluebird males with longer tarsi and tails are more successful at establishing in open areas, where nest-sites are abundant (Duckworth 2006b). In northern populations, dispersive males are those coming from eggs with higher concentrations of testosterone, laid by females exposed to increased competition for nest-sites. Hence, the fact that larger tarsi in male offspring are linked to higher competition for breeding sites in both populations could suggest that maternal effects other than testosterone deposition in the egg might also play a role in shaping offspring phenotype in environments with increased levels of competition.

Chicks from the experimental group exhibited a slightly slower breathing rate than control chicks, but no effect of the experimental manipulation was detected in the tonic immobility test. The behavioural tests used in this study have been proposed as proxies of exploratory behaviour. For example, lower breathing rates have been linked to lower exploration scores in 14-day-old Great Tit nestlings (Fuciková et al. 2009), and exploration is a trait associated with dispersive

behaviour (Clobert *et al.* 2009). Therefore, this result might suggest that in our study population, females exposed to greater competition could produce less exploratory, and presumably less dispersive, offspring. This result would contrast with our interpretation of the effect of the treatment on male offspring tarsus length. However, whether these proxies of exploration measured during early life are associated with dispersive behaviour during adult life has not been investigated in depth yet.

Nestlings from the experimental group exhibited lower, but marginally significant, survival probability than control nestlings; 33% of experimental nestlings died vs. 3% of control nestlings. Faster growth trajectories exhibited by experimental chicks during the rapid growth phase could have negatively influenced survival probability (Metcalfe & Monaghan 2003, Dmitriew 2011) through the action of oxidative stress (Smith *et al.* 2016) and/or reduced immune response (Mauck *et al.* 2005, Pitala *et al.* 2010), among other possibilities. Further studies are required to confirm the potential effect of the experimental treatment (and its underlying mechanisms) on offspring survival.

Results of studies assessing the effect of competition on the deposition of yolk testosterone concentration are inconsistent among species (Smiseth *et al.* 2011), showing an increment (Mazuc *et al.* 2003, Pilz & Smith 2004, Hargitai *et al.* 2009, Bentz *et al.* 2013), no effect (Bentz *et al.* 2018) or effects on other androgens (i.e. androstenedione) instead of testosterone (Remeš 2011). Interestingly, even different populations of the same species may exhibit different patterns of yolk testosterone variation (Albers *et al.* 2017; e.g. Navara *et al.* 2006, Bentz *et al.* 2016b). Taken together, results from previous studies and our own findings suggest that ecological differences among populations may play a determinant role in the specific maternal effect and the key triggering stimulus, highlighting the sensitivity of maternal responses to a range of environmental challenges. In our study, the experimental manipulation of female competition for nest-sites did not affect yolk testosterone concentration, but did impact nestling growth trajectory and breathing rate. Differences between our results and previous findings for northern populations of Western Bluebirds suggest that differences in ecological conditions play a key role in determining the stimuli that can trigger maternal effects, the mechanisms underlying those effects and their results on the offspring phenotype.

AUTHOR CONTRIBUTIONS

América Hernández: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal). **Alejandro Hernández-Martínez:** Investigation (equal); writing – review and editing (equal). **Leticia Nicolás:** Investigation (supporting); supervision (supporting); writing – review and editing (equal). **Roxana Torres:** Conceptualization (equal); supervision (equal); writing – review and editing (equal). **Bibiana Montoya:** Conceptualization (equal); supervision (equal); investigation (equal); methodology (equal); writing – review and editing (equal).

ETHICAL NOTE

Permission to conduct the study was granted by La Malinche National Park Administration Office and SEMARNAT (SGPA/DGVS/9162/19). This research complies with the current laws of Mexico and the Animal Behaviour Guidelines for experimentation with animals.

FUNDING

CONFLICT OF INTEREST

We declare we have no competing interests.

Data Availability Statement

The datasets generated and/or analysed during the current study are available as electronic supplementary material. Additionally, we included as supplementary material, the statistical analyses of the effects of the experimental manipulation on female reproductive performance.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Competitive events during nest building influence offspring growth and behaviour, but not testosterone deposition in the egg yolk in Western Bluebirds.

Fig. S1. Variation of yolk testosterone concentration (mean ± se) with laying sequence in Western Bluebird. Sample sizes of biopsied eggs according to laying order are: in the experimental group ($n = 16$), first eggs = 4, second eggs = 5, third eggs = 4 and fourth eggs = 3; in the control group ($n = 10$), first eggs = 2, second eggs = 2, third eggs = 3 and fourth eggs = 3.

Appendix S1: Competitive events during nest building influence offspring growth and behaviour, but not testosterone deposition in the egg yolk in Western Bluebirds

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Female reproductive performance

The effect of the experimental manipulation on laying probability was evaluated by fitting a Generalized Linear Model (GLM), with binomial error distribution and *logit* link function (package *lme4*; Bates *et al.* 2015). Models evaluating the effect of the treatment on laying date and latency from the first intrusion trial to laying (i.e., laying latency) were fit using independent Linear Models (LM) including the treatment as the fixed effect. The influence of the treatment on individual egg volume was analysed by fitting a Generalized Linear Mixed Model (GLMM) with normal error distribution, including in each model nest identity as the random factor, treatment as the fixed effect, laying date as a covariate, and the interaction between the treatment and laying date. The effect of the treatment on clutch size was analysed using GLM with Poisson error distribution, a *log* link function.

Laying probability ($z = 1.09$, df = 48, $P = 0.274$), laying date ($F_{1,31} = 0.14$, $P = 0.712$), laying latency ($F_{1,28} = 1.27$, $P = 0.269$), or clutch size ($z = -0.14$, df = 30, $P = 0.885$) were not influenced by the experimental manipulation. Egg volume did not differ between groups ($F_{1, 12.92} = 0.06$, $P = 0.807$), and it was not explained by laying date ($F_{1, 13.35} = 0.44$, $P = 0.516$), or the interaction between treatment and laying date ($F_{1, 10.95} = 1.29$, $P = 0.280$).

Yolk testosterone and laying order
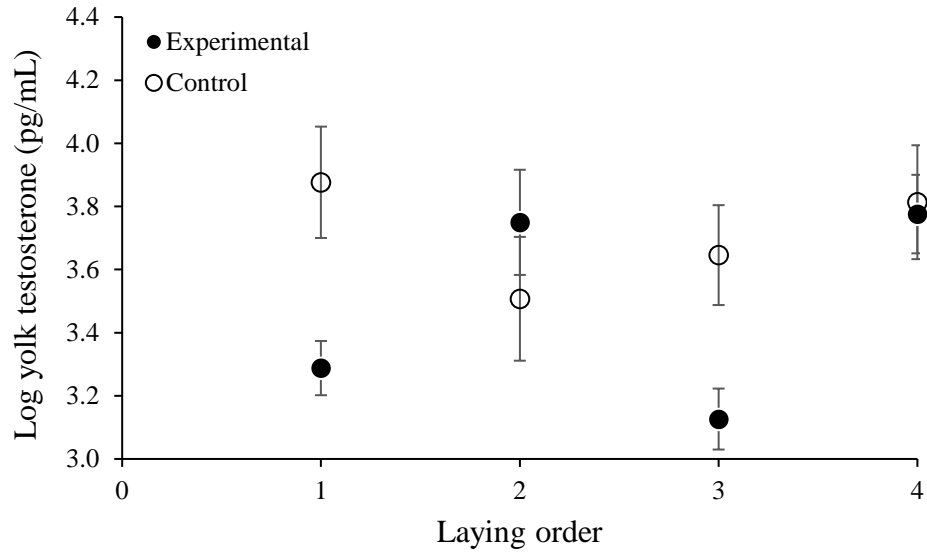
Figure S1. Variation of yolk testosterone concentration (mean ± se) with laying sequence in Western Bluebird. Sample sizes of biopsied eggs according to laying order are: in the experimental group (n = 16), first eggs = 4, second eggs = 5, third eggs = 4, and fourth eggs = 3; in the control group (n = 10), first eggs = 2, second eggs = 2, third eggs = 3, and fourth eggs = 3.

CAPÍTULO 2

Does maternal competition impact nestling development through changes in corticosterone and oxidative damage to lipids?

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ABSTRACT

The developmental and parental environment can exert important effects on the phenotype of the developing offspring. Corticosterone (CORT), the main glucocorticoid in birds, is considered an important mediator of energy mobilization between different vital functions and stages of the life cycle. Although energy mobilization may result advantageous for some life-history traits, it may also inflict oxidative costs because it promotes an augment in the metabolic rate. In a previous study in Western Bluebirds (*Sialia mexicana*), maternal competition for nest cavities was found to affect chick growth. Offspring of females that experienced higher levels of competition exhibited lower body mass at hatching and ulna length gain during the slow growth phase, but higher body mass gain during the rapid growth period, than nestlings of control females. Here, using chicks of Western Bluebirds as a model of study, we evaluated whether increased circulating baseline CORT and oxidative damage to lipids (lipid peroxidation) during early development are the mechanisms that link maternal competition over breeding sites during nest construction with the post-hatching growth rate of the offspring.

Likewise, we assessed whether maternal competition affects differently CORT and lipid peroxidation levels in male and female nestling. We found that manipulation of maternal competition during nest construction did not affect nestlings CORT or lipid peroxidation levels at age 3 days post-hatching, while structural body size gain was linked to these physiological parameters. Only control male chicks showed a positive relationship between CORT levels and structural size. Moreover, control male chicks with high lipid peroxidation levels exhibited lower tarsus length at fledging. In experimental male nestlings, no associations were found between CORT and lipid peroxidation with body growth. Hence, our results do not support the idea that energy mobilization (CORT) or its subproducts (lipid peroxidation) link maternal competition over breeding sites with offspring growth rate. Interestingly, our study suggests that prenatal environment has a sex-specific influence on nestlings' growth since higher CORT titers favor structural growth, however, this effect is condition-dependent, and entails costs in body maintenance, such as oxidative damage to lipids.

INTRODUCTION

Early development comprises a series of energetically demanding events that are regulated through endocrine processes (Wingfield et al. 1998). Glucocorticoids are proposed as the main mediators of energy requirements, since they redirect the energy available to cope with demanding conditions (Landys et al. 2006). These hormones result from the action of the hypothalamic-pituitary-adrenal (HPA) axis and their concentration plays an important role during predictable and energetically demanding periods such as embryonic and post-natal growth (i.e., life history stages and/or environmental signals that occur at a certain point in the life cycle, Wingfield and Romero 2010). For example, in developing mammals, glucocorticoid levels increase near birth and play a key role in organ maturation during the fetal period (Fowden and Forhead 1998). In developing amphibians, glucocorticoids determine the timing of metamorphosis (Kulkarni and Buchholz 2014). In some avian species, increased levels of glucocorticoids promote higher growth rates (Tilgar et al. 2017). Interestingly, glucocorticoid circulating levels can also increase in response to unpredictable environmental challenges (Sapolsky et al. 2000), promoting the reallocation of resources from self-maintenance to functions that promote survival in the short term (Wingfield et al. 1998). For instance, higher

glucocorticoid titers can act as a behavioral modulator to promote food intake, through begging rate and an aggressive behavior, which could facilizing growth rate, or favor locomotor activities to escape from the site where the unpredictable event prevails (Kitaysky et al. 2001, Wingfield and Romero 2010). However, although the trade-offs between maintenance and survival could buffer the negative effects of environmental adversities in a specific life-stage, they may carry costs in subsequent stages (Monaghan 2008). In chicks, unpredictable and detrimental environmental challenges such as food shortage (Corbel and Groscolas 2008, Honarmand et al. 2010), parasite infestation (Lobato et al. 2008), or harsh weather conditions (Lynn and Kern 2014, Crino et al. 2020) increase corticosterone (CORT) levels, the main glucocorticoid in birds. Moreover, in altricial birds, unpredictable events experienced by parents may also affect the developing young through changes in CORT levels. For example, the time that female spends outside the nest is positively related to nestlings circulating CORT (Rensel et al. 2010, Lynn and Kern 2014, Dupont et al. 2021). During environmental perturbations, the potential adaptive role of increasing CORT levels is to trigger an emergency life-history stage that deviates resources from other functions to enhance survival (Wingfield et al. 1998), potentially favoring individual's fitness in the long-term (i.e., CORT–Adaptation Hypothesis; Bonier et al. 2009b). However, when this emergency life-history stage that deviates resources from other functions to enhance survival (Wingfield et al. 1998), potentially favoring individual's fitness in the long-term (i.e., CORT–Adaptation Hypothesis; Bonier et al. 2009b). However, when this emergency life-history stage is prolonged over time, detrimental fitness consequences can emerge (Wingfield and Romero 2010). Accordingly, studies in different avian species have shown that nestling condition is negatively linked to CORT levels (Saino et al. 2003, Pravosudov and Kitaysky 2006, Love and Williams 2008).

Chronically elevated CORT levels (i.e., days to weeks) have been shown to suppress growth (Wada and Breuner 2008, Müller et al. 2009) and immune response (Stier et al. 2009), while increasing metabolic rate (Spencer and Verhulst 2008, Schmidt et al. 2012), so promoting oxidative unbalance (Hall et al. 2010, Stier et al. 2014) that may put at risk biomolecules integrity (i.e., lipids, proteins, nucleic acids) (Bayr 2005), and ultimately organisms' fitness (i.e., CORT–Fitness Hypothesis; Bonier et al. 2009a). Oxidative stress is an unbalance between the number of reactive oxygen species (ROS) and the antioxidant defenses, in favor of the former

(Monaghan et al. 2009). Reactive oxygen species are metabolic byproducts regularly produced during the process of transduction of nutrients into energy available for vital processes such as growth (Hall et al. 2010, Stier et al. 2014). CORT secretion can induce oxidative stress by promoting energy mobilization, and hence, the release of ROS during associated metabolic processes (Sapolsky et al. 2000, Costantini et al. 2011), like accelerated growth (or catch-up growth) (Geiger et al. 2012, Losdat et al. 2013, Reichert et al. 2015). Therefore, any demand imposed on the metabolic machinery to increase energy production may increase oxidative stress potentially leading to detrimental consequences.

Secondary cavity-nesting birds compete intensively with other individuals to acquire a nesting-site to reproduce because they cannot excavate their own (Newton 1994), so suitable cavities are a limited resource. Although in several species, males have a principal role at stablishing and defending the territory, females also compete for resources (Cain and Ketterson 2013, Krieg and Getty 2020). Competitive confrontations may influence females' physiology and behavior with potential cascading effects on the offspring (i.e., maternal effects), since it can induce changes in the allocation of hormones in the yolk or promote modifications in parental behaviors, with concurrent impacts on nestlings' growth, body maintenance or survival (Bentz et al. 2013, Krieg and Getty 2020). In Western bluebirds (*Sialia mexicana*), experimentally increased competition over breeding sites reduces body mass at age 3 days and ulna growth during the slow growth period (phase near the plateau, 9-15 days post-hatching), but accelerates body mass gain in the rapid growth period (stage of exponential growth, 3-9 days post-hatching) and produces male chicks with longer tarsi at age 15 days (Hernández et al. 2022). Hence, maternal competition over nest-sites influences offspring phenotype during development, but after excluding embryo exposure to higher testosterone levels as a potential mechanism, it is still unclear what could be the underlying physiological process (Hernández et al. 2022).

Here, we aimed to evaluate whether the previously reported effect of increased competition for breeding cavities on offspring growth rate (Hernández et al. 2022) is mediated by the action of CORT levels in the nestlings and the cost of increased oxidative damage. In such a case, we predict that CORT titers and lipid peroxidation levels at age 3 days will be higher and positively associated with growth rate in nestlings from the experimental group (i.e.,

mothers exposed to increased competition) when compared with nestlings from the control group. Additionally, we evaluated whether maternal competition affects differently CORT and lipid peroxidation levels in male and female chicks then, accordingly to our previous growth results, we expect that experimental male nestlings will show higher CORT and lipid peroxidation levels compared to control male nestling, but no differences are expected in female chicks of both groups. This sex difference in our predictions relies on the fact that, in our previous study, experimental male chicks exhibited larger tarsi compared with control nestlings, but no differences were found in the structural growth of female chicks.

METHODS

The study was conducted from April to July of 2019, during the breeding season of Western bluebirds at La Malinche National Park, Tlaxcala, Mexico. Under natural conditions, cavities are mainly present in the mature forest (Sánchez et al. 2007). However, human activities (agriculture, logging, and grazing) and the removal of dead trees reduce the availability of suitable nest sites (Guinan et al. 2008). Hence, the population of Western bluebirds included in this study breeds in nest boxes installed in a young deciduous and coniferous forest (Cuatianquiz and Macías-Garcías 2016). In this population, nest building lasts an average of 14.67 days (± 1.97) and takes place between late March and early May (average in mild–April), and laying occurs from late April to late May (average in early May; Montoya B. unpublished data from 2015-2019).

To manipulate the perceived intensity of competition during the nest building period, before pairs were settled in their territories, we installed an additional nest box 5 to 10 m apart from each focal nest box (i.e., boxes that were occupied by a breeding pair during the last three reproductive seasons) to increase nesting-site availability. Fifty nest boxes were included in the study, all boxes were visited twice a week to identify nest building attempts and assign them to one treatment. In the experimental group (i.e., high competition; n = 26 nests), the additional nest box was removed, and a series of simulated territorial intrusions were performed. Each simulated intrusion consisted of the presentation of a 3D model and a playback with vocalizations of a house wren (*Troglodytes aedon*), one of the main competitors for breeding cavities in this population (Hernández et al. 2022). This manipulation aimed to modify females'

perception of nesting-cavity competition. In the control group (n = 24 nests), the additional box was not removed, and the intrusion trial consisted of exhibiting a 3D model and a playback of a red warbler (*Cardellina rubra*), a ground-nesting species that does not compete for nest-cavities (Hernández et al. 2022). In the experimental and the control group, once the decoy was placed on the top of the focal nest box and the female was visually localized, the playback was reproduced for 10 minutes. The time of arrival of the females after the decoy was placed was positively related to the laying date in the experimental group, so the control females took longer to arrive at the nest as the breeding season progressed (experimental = 14.04 ± 1.65 min, control = 23.12 ± 1.66 min; treatment × laying date: $\beta = 0.95 \pm 0.30$, $F_{1, 19.71} = 10.03$, $P = 0.005$). All nests included in the experiment were followed twice a week to determine the laying date. In the control group, the additional nest box installed before the breeding season was removed after clutch completion to maintain similar conditions in both groups during the incubation period and post-hatching parental care.

Nests were checked daily around the estimated hatching date to determine when it occurred. At ages 3, 9, and 15 ± 1 day post-hatching, we measured nestlings' skull (i.e., skull and bill), tarsus, and ulna length using a caliper (± 0.02 mm) and body mass with a digital balance (± 0.01 g). Each nestling was marked with nontoxic and water-resistant nail polish on the toenail, to maintain individual identification for up to 15 days, when fledglings were ringed with a unique combination of colored and numbered polymethylmethacrylate leg bands (Interrex). To assess plasma CORT levels and lipid peroxidation, nestlings were blood sampled at age 3 days, extracting 50 µL of blood from the jugular vein. Preliminary tests were performed to confirm that blood samples were obtained within three minutes after handling started. Blood samples were centrifuged at 10000 g for 15 min to separate plasma from red blood cells, and those fractions were stored at -80° C until analyses.

Corticosterone concentration was determined in samples of 20 µL plasma. Total steroids were extracted using 1 mL of diethyl ether twice until evaporation, and finally, adding 1 mL methanol following a protocol published elsewhere (López-García et al. 2013). Extracts were re-suspended in 50 µL of deionized water. Hormone level was determined through a corticosterone enzyme immunoassay kit (DRG International, Inc.), and measured at 450 nm of absorbance, with correction at 630 nm using a microplate photometer (Multiskan FC, Thermo

Fisher Scientific). Obtained values were interpolated in a reference curve of corticosterone standards provided together with the kit. The coefficients of variation were 5.96 % within plates and 5.17 % among plates. Throughout the manuscript, results are expressed as pg of corticosterone/mL.

Nestlings' lipid peroxidation, was estimated by the thiobarbituric acid reactive substances assay (TBARS), previously used in this species (Montoya et al. 2020). Briefly, 100 µL of 10% trichloroacetic acid was added to 100 µL of 5 µL of plasma diluted in 95 µL of deionized water, this solution was centrifuged at 3000 g for 10 min, and 180 µL of the supernatant was recovered and added to 100 µL of thiobarbituric acid reagent (0.375% TBA and 2% acetic acid). This mix was incubated at 92° C for 45 min, and then, placed on ice for 5 min. The optical density of the resulting product was measured at 530 nm using a microplate photometer (Multiskan FC, Thermo Fisher Scientific) and interpolated to a standard curve of 1, 1, 3, 3-tetramethoxypropane (Sigma-Aldrich, St. Louis, MO). Intra-plate variation was 1.73 % and inter-plate variation was 0.88 %. Lipid peroxidation was estimated as nM MDA equivalents.

Statistical Analysis

Analyses were performed using R statistical software, version 4.1.0 (R Development Core Team 2018) and package *lme4* (Bates et al. 2015). Final models were obtained by stepwise backward deletion of non-significant terms ($P > 0.05$). Sample sizes may differ slightly among analyses due to nestling mortality or missing data (e.g., insufficient sample volume for quantification of CORT and lipid peroxidation levels). Prior to statistical analyses, CORT titers and lipid peroxidation were ln transformed to meet normality assumptions. To assess the effect of the experimental manipulation on nestlings' CORT titers and lipid peroxidation, two independent Generalized Linear Mixed Model (GLMM) with normal error distribution were fitted. Models included nest identity as a random factor, treatment as a fixed effect, laying date as a covariate, and the interaction between the treatment and laying date. Additionally, in models of lipid peroxidation, CORT titers were also included to control the potential influence CORT.

To evaluate the association of CORT titers and lipid peroxidation with nestlings' body mass at age 3 days, body mass gain during rapid growth period, ulna length gain during slow growth period, and tarsus length at age 15 days we used independent GLMMs with normal error

distribution. Before the analysis, body mass gain from 3–9 days post-hatching (i.e., rapid growth period) was calculated as the difference between the initial body mass (day 3) from final body mass (day 9), divided by the initial body mass (day 3). Similarly, to calculate the ulna length gain during slow growth period (i.e., 9-15 days post hatching) the difference of ulna length at days 9 and 15 was obtained, and then divided between the measure recorded at day 9. On the one hand, models included treatment as a fixed effect, CORT titers, and the interaction between treatment and CORT titers. On the other hand, to assess the influence the oxidative damage on nestling's growth, models included treatment as a fixed effect, lipid peroxidation levels, CORT titers, and the interaction between treatment and lipid peroxidation. All models included nest identity as a random factor. Because the experimental treatment was previously found to differentially affect tarsus length at age 15 days depending on the nestling's sex (GLMM with normal error distribution: $\beta = 1.71 \pm 0.45$, $F_{1,\ 32.74} = 14.20$, $P < 0.001$, Hernández et al. 2022), to explore the association of CORT titers and lipid peroxidation levels with tarsus length at age 15, independent models for each sex were fitted.

RESULTS

The experimental manipulation did not influence CORT titers or lipid peroxidation levels of chicks at age 3 days (Figure 1). CORT titers of chicks from the control and experimental groups did not differ ($F_{1,\ 23.23} = 1.88$, $P = 0.18$, Figure 1a), and were unrelated to hatching date ($F_{1,\ 26.84} = 2.83$, $P = 0.10$), or the interaction between treatment and hatching date ($F_{1,\ 23.17} = 0.08$, $P = 0.78$). Lipid peroxidation levels were unrelated to the experimental treatment ($F_{1,\ 19.62} = 0.98$, $P = 0.34$, Figure 1b), CORT titers ($F_{1,\ 52.78} = 3.19$, $P = 0.08$), or the interaction between treatment and hatching date ($F_{1,19.04} = 1,14$, $P = 0.30$). However, levels of lipid peroxidation were lower in chicks that hatched later in the season ($\beta = -0.05 \pm 0.02$, $F_{1,\ 22.04} = 6.42$, $P = 0.02$).

Body mass at age 3 days was not associated with the treatment, CORT titers, lipid peroxidation levels, or the two-way interactions between the treatment and CORT levels and the treatment and lipid peroxidation levels (Table 1). Body mass gain during the rapid growth period did not differ between experimental and control nestlings, and it was unrelated to lipid peroxidation levels, or hatching date. Moreover, the interaction between treatment and CORT levels or treatment and lipid peroxidation levels did not explain variation in body mass gain

(Table 1). However, body mass gain showed a negative relationship with CORT titers: chicks with higher body mass gain had lower CORT levels ($\beta = -0.07 \pm 0.03$, Table 1, Figure 2). CORT titers and lipid peroxidation levels, as well as the interactions of each of them with the treatment did not influence ulna length gain during the slow growth period. However, experimental chicks exhibited lower ulna length gain compared with nestlings from control females, as has been described previously (Hernández et al. 2022).

In male control chicks, tarsus length at age 15 days was positively associated with CORT levels (Table 2, Figure 3a), while in nestlings of the experimental group CORT titers were not associated with tarsus length (Table 2, Figure 3a). Additionally, male nestlings from the control group presented a negative association between tarsus length and lipid peroxidation (Table 2, Figure 3b), yet no association was found in experimental chicks. Interestingly, female nestlings with high lipid peroxidation levels exhibited shorter tarsus (Table 2, Figure 3c) regardless of treatment group. In females, CORT levels and the interactions of treatment with CORT and lipid peroxidation levels were unrelated to tarsus length.

DISCUSSION

Our experimental study reveals that maternal competition during nest construction in Western bluebird does not affect nestlings CORT and lipid peroxidation levels, two important indicators of self-maintenance (Costantini et al. 2011). Although nestling body mass and ulna length were not linked to CORT or lipid peroxidation, tarsus length was associated with CORT and lipid peroxidation, particularly in male control nestlings and female chicks.

We predicted a positive effect of the experimental treatment (i.e., increased competition for nesting-sites) on nestlings CORT levels, since parental attendance and provisioning effort could be affected by environmental conditions. This assumption relies on previous studies reporting increased CORT levels in nestlings associated with a reduction in nest attendance or provisioning effort by parents (Rensel et al. 2010, Ospina 2017). Moreover, in a previous study, we found that experimentally increased competition for nesting sites had a negative effect on chicks' body mass at the age of 3 days (Hernández et al. 2022), and nestling poor condition has been linked to high CORT levels in different species (Saino et al. 2003, Pravosudov and Kitaysky 2006, Love and Williams 2008). However, contrary to our predictions, the

experimental treatment did not affect nestling´s CORT levels. Hence, competition over breeding cavities does not exert a negative impact on offspring via increased CORT levels. A potential explanation for these results that needs further evaluation is that at age 3 days, the HPA axis is at an immature stage, which may prevent a rise in CORT levels in response to environmental stimulation (i.e., parental unattendance resulting from higher competition) (Wada et al. 2007, Wada 2008). No differences in CORT concentration between groups could also indicate that if experimental nestlings face temporary nutritional restrictions, as suggested by their lower condition at day 3 post-hatching, they could decrease CORT secretion, hence avoiding the negative effects of maintaining chronically increased CORT levels, as found in Cory's shearwaters (*Calonectris diomedea*; Fairhurst et al. 2012). For instance, in Eastern bluebird (*Sialia sialis*) parents coordinate feeding trips to offspring when breeding at higher densities of a nesting competitor (Siefferman and Burdick 2020). However, in this study we do not have data to evaluate the behavioral traits of progeny and parents.

Body mass gain during rapid growth period exhibited a negative relationship with CORT titers, but this association did not depend on the experimental treatment. This negative association between body mass and CORT titers has been reported for other altricial species (Wada et al. 2007). This result may indicate that such elevated CORT titers may be deviating energetic resources from growth to other functions during early stage of nestlings' development, and hence, chicks with higher CORT levels are in poor condition. Interestingly, no links were found between CORT and lipid peroxidation levels, so oxidative damage to lipids does not appear to be a proximate cost of holding high CORT titers, in this case. Contrastingly, ulna length gain during slow growth period was not associated with CORT and lipid peroxidation levels at age 3 days. However, tarsus length at age 15 was positively associated with CORT titers, only in control males. Taken together these results suggest the occurrence of a condition-dependent effect of CORT titers in body growth, which would be consistent with predictions derived from the CORT–Fitness Hypothesis (Bonier et al. 2009a).

We predicted a positive effect of the experimental treatment (i.e., competition for nesting-sites on lipid peroxidation levels) on nestlings' lipid peroxidation levels, yet no effects of the experimental treatment were found. It would be relevant to evaluate if the absence of association found between manipulation of maternal competition and nestlings' lipid damage

levels was mediated by an increase in the antioxidant capacity, since in this work we did not assess the antioxidant defense. This result is consistent with the absence of an effect of the experimental manipulation on CORT levels, as we had hypothesized lipid peroxidation elevation as a proximate cost of holding higher CORT titers. Consistently with results on the association between CORT and tarsus length, lipid peroxidation levels were negatively related to the tarsus length of male fledglings from the control group, while no association was identified in male fledglings from the experimental group. Interestingly, females with higher lipid peroxidation levels exhibited shorter tarsi at age 15 days, this association is equivalent to that found in males from the control group, yet in females, there was no effect of the treatment on the association between lipid peroxidation and tarsi length.

The potential influence of CORT and oxidative damage to lipids in the developmental strategies of the nestlings appears to be sex-specific. Sex-specific mechanisms can promote differences in nestlings' growth rate associated with CORT and lipoperoxidation titers due to sex differences in transport proteins (i.e., corticosterone binding globulins), glucocorticoid receptor expression (Hayward et al. 2005), HPA axis activity (e.g., Lui et al 2001), metabolic responses (Spencer and Verhulst 2008), or antioxidant defenses (Salomons et al. 2009, Giordano et al. 2015). Moreover, these negative associations found between lipid peroxidation and tarsus growth provide support for the idea that oxidative stress constrain growth (besides being a cost of catch up; Geiger et al. 2012). Nevertheless, results about male nestlings' growth should be interpreted with caution, due to the small sample size available for the analysis. Then, further studies are needed to improve our understanding on the sex-specific impacts of prenatal condition on chicks' fitness, reproduction and survival.

In conclusion, we found no support for our predictions that CORT titers and levels of oxidative damage to lipids during early development are the mechanisms linking maternal competition over breeding sites with changes in nestling growth rate trajectories. However, our study suggests that, in Western bluebirds, maternal environment influences offspring growth through sex-specific effects in CORT and lipid peroxidation levels of chicks. Moreover, CORT levels appear to be linked with different growth strategies in a condition-dependent way as predicted by the CORT–Fitness Hypothesis.

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FIGURES
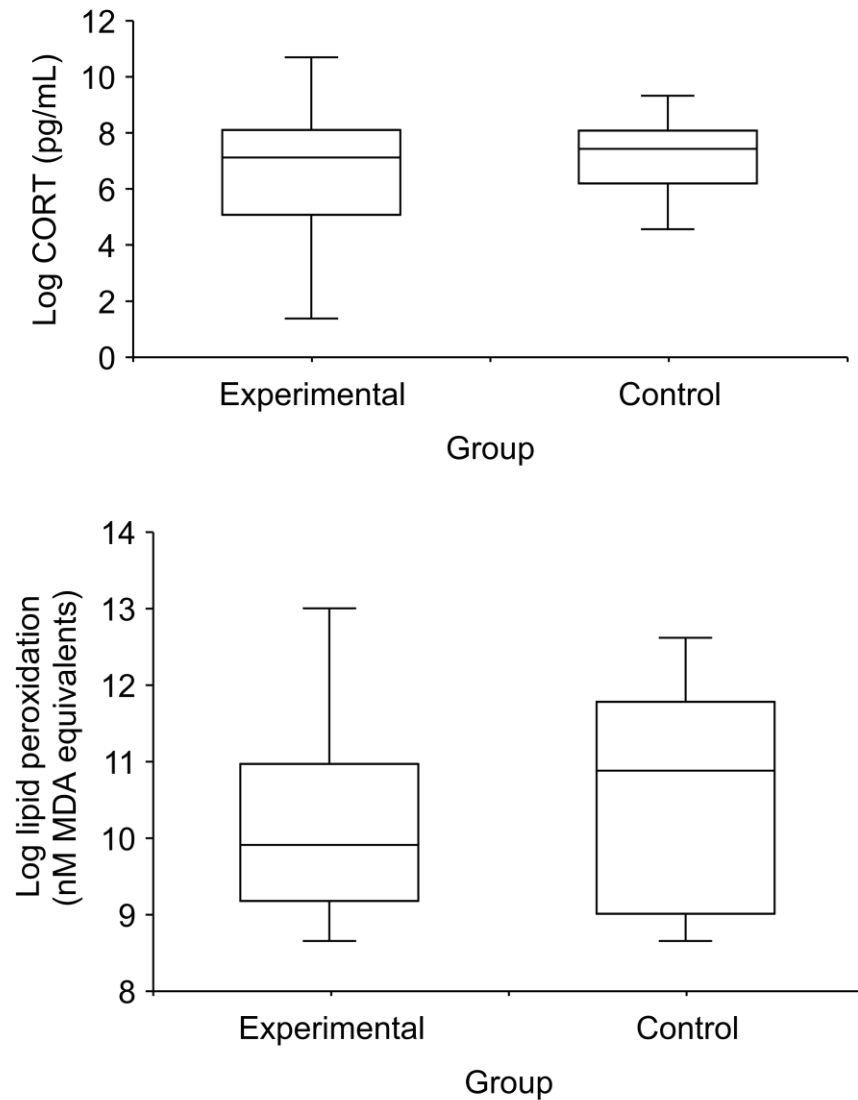
Figure 1. Effect of experimentally increased maternal competition during nest construction on (a) CORT and (b) lipid peroxidation levels (values transformed to ln) in Western bluebird nestlings at age 3 days. To manipulate competition levels females in the experimental group were exposed to territorial intrusions with a decoy and vocalizations of a competitor species and a nest box near their nest was removed, while females in the control group were exposed to territorial intrusions with a decoy and vocalizations of a non-competitor species and the nearest nest box was not removed.
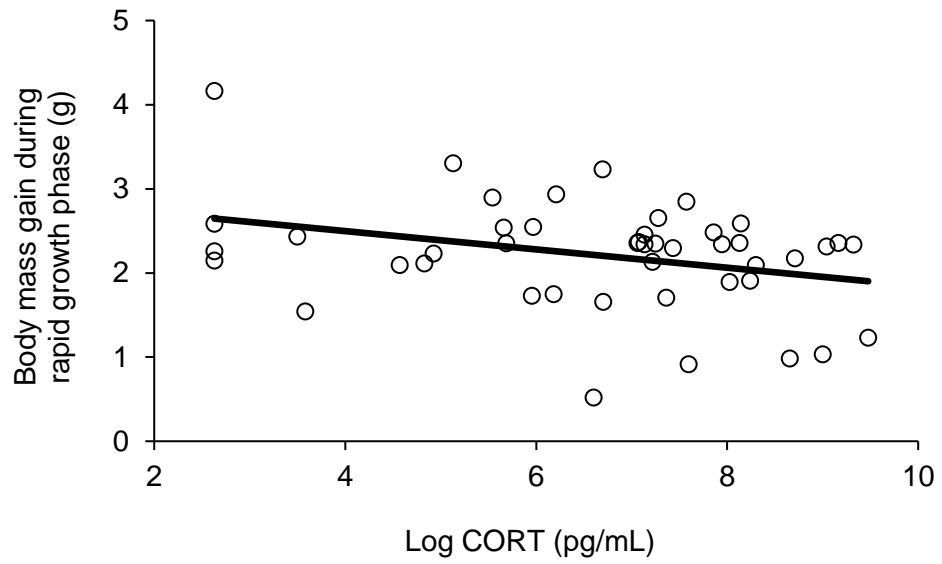
Figure 2. Relationship between nestlings' CORT titers at age 3 days and offspring body mass gain during rapid growth phase at age 3-9 days in Western bluebirds.
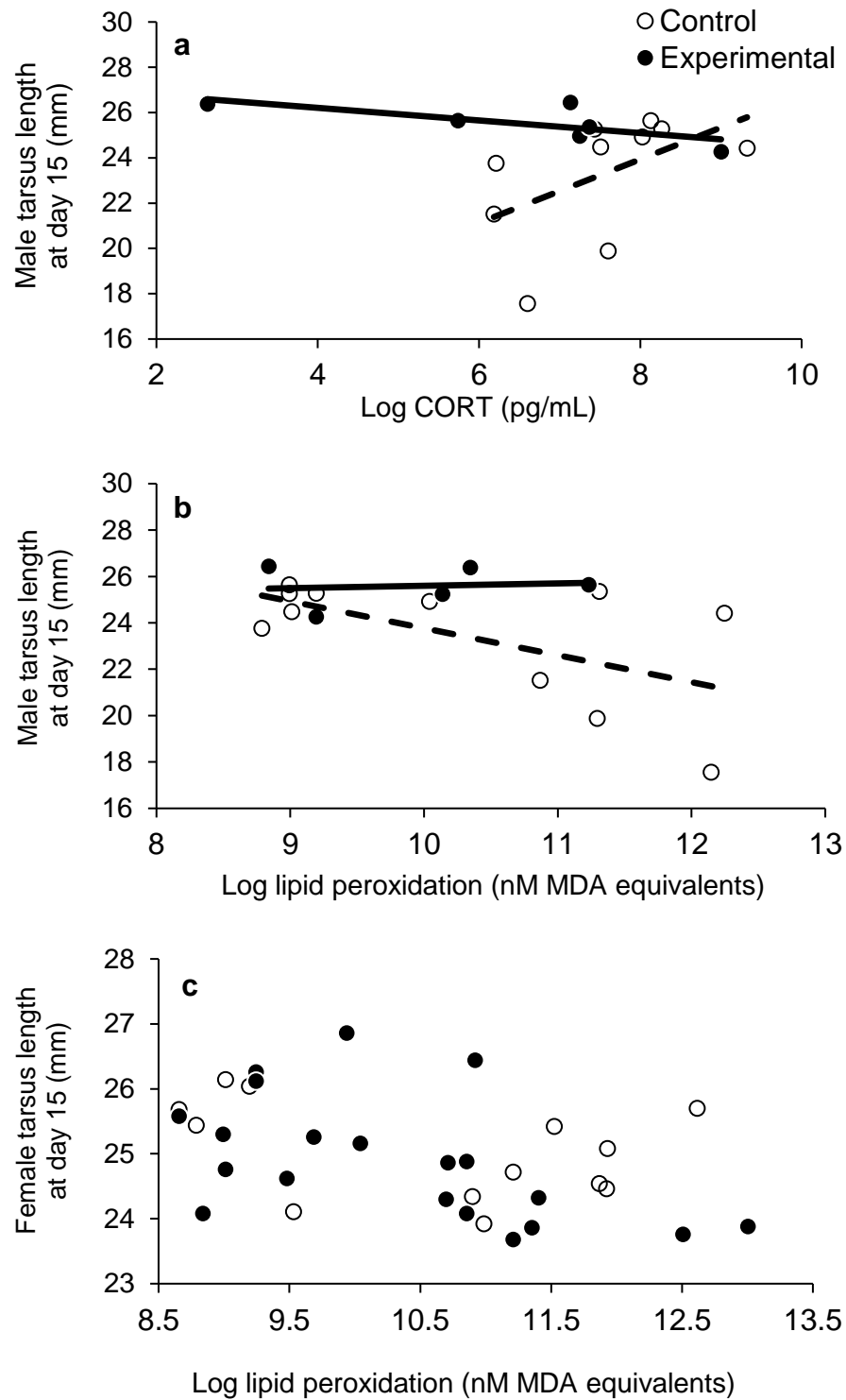
Figure 3. Effects of the maternal competition on (a) the association between CORT titers and tarsus length at age 15 days in Western bluebird male nestlings, (b) the association between lipid peroxidation levels and tarsus length at 15 days in male nestlings, and (c) the association

between lipid peroxidation levels and tarsus length at 15 days in female nestlings. CORT titers and lipid peroxidation levels were ln transformed. Experimental nestlings descended from females that were experimentally exposed to a decoy and vocalizations of a competitor species for cavities and the nearest nest box to their nest was removed, whereas control nestlings descended from females that were exposed to simulated territorial intrusions with a decoy and vocalizations of a non-competitor and nest sites were not reduced.

TABLES

Table 1. Association of initial body size and body size gain of offspring from experimental and control nests with CORT titers and lipid peroxidation levels. *F* and *P* values in bold indicate variables included in the final model after excluding non-significant terms.

	Estimate ± SE	*F*	DF	*P*
CORT TITERS				
Body mass at age 3 days (n = 67)				
Treatment	-0.07 ± 0.79	0.007	22.44	0.94
CORT titers	0.11 ± 0.09	1.18	51.07	0.28
Treatment × CORT titers	-0.03 ± 0.28	0.01	46.94	0.91
Body mass gain during rapid growth period (n = 59)				
Treatment	0.17 ± 0.27	0.42	21.12	0.53
CORT titers	**-0.07 ± 0.03**	**6.54**	**39.45**	**0.01**
Treatment × CORT titers	-0.09 ± 0.07	1.74	37.31	0.20
Ulna length gain during slow growth period (n = 51)				
Treatment	**-0.14 ± 0.55**	**6.24**	**18.42**	**0.02**
CORT titers	-0.01 ± 0.01	0.66	41.69	0.42
Treatment × CORT titers	0.03 ± 0.03	1.12	38.82	0.30
LIPID PEROXIDATION LEVELS				
Body mass at age 3 days (n = 56)				
Treatment	0.02 ± 0.93	0.0005	19.21	0.98
CORT titers	0.04 ± 0.13	0.08	38.32	0.77
Lipid peroxidation levels	-0.04 ± 0.20	0.04	52.99	0.85
Treatment × Lipid peroxidation levels	0.44 ± 0.41	1.13	42.79	0.29
Body mass gain during rapid growth period (n = 50)				
Treatment	0.05 ± 0.28	0.04	19.22	0.85
CORT titers	**-0.07 ± 0.03**	**6.54**	**39.45**	**0.01**
Lipid peroxidation levels	-0.02 ± 0.06	0.19	36.95	0.67
Treatment × Lipid peroxidation levels	-0.01 ± 0.12	0.01	35.76	0.91
Ulna length gain during slow growth period (n = 45)				
Treatment	**-0.14 ± 0.06**	**6.24**	**18.42**	**0.02**
CORT titers	-0.009 ± 0.01	0.66	41.69	0.42
Lipid peroxidation levels	0.02 ± 0.02	0.67	40.81	0.42
Treatment × Lipid peroxidation levels	-0.07 ± 0.04	2.85	39.29	0.09

Table 2. Association of tarsus length at age 15 days of offspring from experimental and control group associated with nestlings' CORT titers and lipid peroxidation levels in males and females. *F* and *P* values in bold indicate variables included in the final model after excluding non-significant terms.

	Males				**Females**			
Tarsus length-CORT titers	**(n = 16)**				**(n = 34)**			
	Estimate ± SE	*F*	df	*P*	Estimate ± SE	*F*	df	*P*
Treatment	15.33 ± 4.11	13.91	8.26	0.005	-0.18 ± 0.38	0.21	18.54	0.65
CORT titers	1.54 ± 0.42	5.07	8.69	0.05	-0.04 ± 0.06	0.33	24.42	0.57
Treatment × CORT titers	**-1.82 ± 0.56**	**10.51**	**8.69**	**0.01**	-0.22 ± 0.17	1.63	19.44	0.22
Tarsus length-lipid peroxidation levels	**(n = 14)**				**(n = 31)**			
	Estimate ± SE	*F*	df	*P*	Estimate ± SE	*F*	df	*P*
Treatment	-23.57 ± 11.84	3.96	8.99	0.08	-0.28 ± 0.37	0.58	15.44	0.46
Lipid peroxidation levels	-1.42 ± 0.43	0.02	8.16	0.89	**-0.25 ± 0.10**	**5.88**	**29.36**	**0.02**
CORT titers	0.78 ± 0.31	6.19	2.86	0.09	-0.09 ± 0.07	1.75	21.83	0.19
Treatment × Lipid peroxidation levels	**2.67 ± 1.19**	**4.96**	**8.98**	**0.05**	-0.28 ± 0.20	1.85	21.02	0.19

Incubation temperature influence maternally-derived yolk testosterone transformation in eggs of the Japanese quails

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ABSTRACT

In birds, changes in hormone level deposition by females into the eggs in response to current environmental conditions can impact offspring phenotype. Although less studied, environmental conditions can also influence a female's incubation behavior, which might play a role in regulating embryo exposure to maternal hormones. Moreover, recent literature suggests that the embryo could participate in hormone metabolism to avoid the effects of testosterone exposure during early development. Here, we tested the hypothesis that both variations in the female deposition of testosterone in the yolk and incubation temperature determine exposure to testosterone metabolites during early embryo development. In the Japanese quails (*Coturnix japonica*), we experimentally increased yolk testosterone and incubated eggs at 38° C or 36° C temperatures (within the natural range) and analyzed testosterone, 5α-dihydrotestosterone, and estradiol titers at day 4 of incubation. We found that eggs with experimentally increased testosterone concentration and those incubated at 36° C showed higher 5α-dihydrotestosterone levels than eggs in the control group incubated at the same temperature and experimental and

control eggs incubated at 38° C, independently of whether the presence of an embryo was observed or not. Testosterone and estradiol titers were not affected by incubation temperature, increased testosterone treatment or the presence of an embryo. Hence, we have no evidence that during the first 4 days of incubation the embryo may play a role in the metabolism of testosterone of maternal origin titers. Our study suggests that in the Japanese quail, variation in incubation temperature influences testosterone metabolism to 5α-dihydrotestosterone, and thus, may act as a maternal effect pathway for shaping adaptive phenotype.

INTRODUCTION

In birds, as well as other vertebrates, hormonal-mediated maternal effects are proposed as a mechanism by which females can adjust offspring phenotypes to prevailing ecological and social conditions, with steroid hormones the main inducer of phenotypical changes (Groothuis et al. 2005). The most parsimonious hypothesis about steroids accumulation in eggs, the physiological epiphenomenon hypothesis, states a positive relationship between a female's circulating levels and yolk hormone concentration (Groothuis and Schwabl 2008, but see Williams et al. 2004). This hypothesis has received support from empirical studies showing that an increase in female circulating levels of testosterone, for instance, in response to environmental challenges correlates with a higher deposition of androgens into the egg yolk, and has consequences in offspring phenotype (Schwabl 1993, Clotfelter et al. 2004, Jawor et al. 2007). Testosterone is one of the key hormones proposed to link the maternal environment with variation in the phenotype of the offspring, modifying several traits like growth, immune response, or survival (Podmokła et al. 2018).

In addition to the differential allocation of hormones to the egg, increased levels of female circulating testosterone in response to environmental challenges can also influence female incubation performance, with potential consequences for the offspring phenotype (Clotfelter et al. 2007, O'Neal et al. 2008, Cain and Ketterson 2013). For example, some studies have shown that females with higher circulating testosterone have less developed brood patches, reach lower incubation temperatures, and spend less time incubating than females with lower levels of testosterone (Clotfelter et al. 2004, Rosvall 2013, Cantarero et al. 2015, De Jong et al. 2016). Incubation temperatures below the optimum can result in slower embryo developmental

rates (Martin et al. 2007), increased metabolic rate at hatching (DuRant et al. 2011), augmented neophobic behaviors (Bertin et al. 2018), alterations in different endocrine axes (DuRant et al. 2010, DuRant et al. 2014), and in some species, incubation temperature plays a critical role in sex determination (Jeyasuria and Place 1998). Interestingly, incubation temperature may also alter hormonal content in the yolk along the embryo development (Elf et al. 2002, Paitz and Bowden 2009, Ding et al. 2012), as some studies in reptiles' temperature-dependent sex determination have suggested (Dorizzi et al. 1991, Crews and Bergeron 1994). However, the possibility that hormones transferred by the mother can be further adjusted through the incubation temperature, to our best knowledge, has not yet been evaluated in birds.

One potential mechanism that may explain the occurrence of modifications in egg hormonal content linked to variation in incubation temperature, is the action of the enzymes aromatase and 5α-reductase. These enzymes depend on temperature and play a role in the metabolism of testosterone (Crews and Bergeron 1994). In eggs, aromatase and 5α-reductase compete for the same substrate, testosterone (from maternal or embryonic origin) (Jeyasuria and Place 1998). At warmer temperatures, aromatase participates in estrogens synthesis from testosterone (Owen-Ashley et al. 2004), while at lower temperatures, 5α-reductase metabolizes testosterone into 5α-dihydrotestosterone, another active androgen involved in sexual differentiation during embryo development (Lance 2009). Once testosterone is converted to 5α-dihydrotestosterone, it cannot be synthesized into estrogens (Owen-Ashley et al. 2004). Interestingly, studies that have assessed the effects of exposure to testosterone *in ovo* and the impact of high circulating testosterone levels during adulthood on individual physiology suggest that testosterone might exert its effects through its conversion to estradiol (i.e., aromatization) instead of having a direct influence (Casagrande et al. 2012). For example, aromatase inhibition in eggs from domestic chicken (*Gallus gallus*) increased antibody production and thymus size in nestlings, which suggests that aromatization act as a pathway of immunosuppression (Simkins et al. 2020). In mature individuals of the common kestrel (*Falco tinnunculus*), experimentally increased titers of estradiol resulted in a rise in oxidative stress levels, while higher 5α-dihydrotestosterone levels did not impact oxidative status, which suggests that testosterone reduces the defense capacity to oxidative stress through its transformation to estrogens, but not by its conversion to a non-aromatizable androgen (Casagrande et al. 2012). However, little is

known about the potential effects of incubation temperature on yolk testosterone metabolism and its consequences on offspring phenotype.

Testosterone deposition in the yolk has been considered to induce predictive adaptive changes in the offspring phenotype (Mousseau and Fox 1998, Groothuis et al. 2005). However, testosterone can also have negative impacts on offspring, suggesting that adaptive consequences can be context-dependent (Marshall and Uller 2007) and opening the question of whether the embryo is a passive receptor of maternally derived hormones or can take an active role in regulating its exposure to them (Moore and Johnston 2009). A growing number of studies on oviparous species suggests that the embryo might control, to a certain degree, its exposure to hormones from the maternal origin (Paitz and Bowden 2011). Two potential mechanisms playing a role in such embryonic regulation are changes in the expression of hormone receptors on extraembryonic membranes (i.e., annexes that are formed from the embryo and that participate in its development; Kumar et al. 2019b), and/or metabolization of maternal yolk testosterone into non-active compounds through the action of embryonic enzymes (Paitz and Bowden 2008; Von Engelhardt et al. 2009; Paitz et al. 2011, Vassallo et al. 2014, Kumar et al. 2019a, Campbell et al. 2020), both mechanisms would occur during the first days of incubation and before the gonad development. However, the potential embryo participation as the regulator of maternally derived hormone exposition has not been explored thoroughly and the potential influence of incubation temperature on them is still unknown.

Japanese quail (*Coturnix japonica*) is a precocial species whose incubation period is approximately 16 days (Huss et al. 2008), the gonadal differentiation occurs between day 5-6 of incubation and aromatase activity has been detected during early development in gonad and brain of the embryo (Carere and Balthazart 2007). Here, we aimed to test the hypothesis that maternal testosterone deposition in the yolk and incubation temperature determine the extent of embryo exposure to maternal androgens during the first days of development of Japanese quails. We performed a full factorial experiment to manipulate testosterone concentration in the yolk (increasing testosterone through an injection) and incubation temperature and we measured testosterone, 5α-dihydrotestosterone and estradiol titers in the embryo at day 4 after laying (i.e., before gonadal maturation, to avoid the confounding effect of endogenous steroid production). To explore the potential direct participation of the embryo on the metabolism of testosterone,

we compared titers of the three hormones in eggs where the development of an embryo was observed and eggs where the presence of an embryo was absent. For Japanese quails, 38° C is within the range of the optimal incubation temperature, and 36° C is the lowest temperature at which chicks can hatch (Romao et al. 2009, Ben-Ezra and Burness 2017). First, we hypothesized that incubation temperature affects the metabolism of testosterone in the egg, presumably through the action of aromatase and 5α-reductase enzymes (Figure 1), with three possible scenarios. (1) If lower incubation temperatures trigger the action of 5α-reductase on yolk testosterone, we expect that increase testosterone eggs and incubated at lower temperatures will have a higher 5α-dihydrotestosterone concentration and lower estradiol concentration compared to eggs from the other three groups (Figure 1). In contrast, if warmer temperatures promote the action of aromatase on yolk testosterone, (2) we expect that increased testosterone eggs and incubated at warmer temperatures will have higher estradiol and lower 5α-dihydrotestosterone concentration compared to eggs from the other three groups (Figure 1). If incubation temperature does not influence yolk hormones, (3) we expect that increase testosterone eggs will exhibit higher testosterone concentration independently of incubation temperature compared to control eggs, and no differences in the levels of 5α-dihydrotestosterone and estradiol in the eggs of experimental and control groups will be found. Second, for the hypothesis about the embryo playing a role in testosterone metabolization in a temperature-dependent way, we expect that scenarios (1) and (2) will occur in eggs with a developing embryo; in contrast, eggs with no developing embryo will show the pattern described in prediction (3; Figure 1).

METHODS

We obtained freshly Japanese quail eggs from a breeding colony maintained at Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Mexico. Eggs were obtained from 40 females and 20 males of 39 to 45 weeks of age. Birds were housed in individual cages (50 x 30 x 20 cm) under a light regimen of 15L:9D. Females and males were provided with 35 and 30 g of commercial quail food (Purina, Codor reproductina HP, Nestlé, Vervey, Switzerland - 21% protein, 3.4% calcium, 0.8% phosphorus), respectively, and water *ad libitum*. Quails were randomly paired by housing the same male in the female's cage for three hours every day for a

week. Freshly laid eggs were collected daily starting from the third day of copulation. Eggs were individually marked with a non-toxic permanent marker (to record the mother´s identity) and weighed with a digital balance (± 0.1 g). After collection, eggs laid by the same female were sequentially assigned to one of four possible treatments, using a full factorial experimental design of 2 × 2: Increased testosterone eggs-incubation temperature at 38° C, increased testosterone eggs-incubation temperature at 36° C, control eggs-incubation temperature 38° C, and control eggs-incubation temperature 36° C (see details of each treatment below). Overall, the experiment included 161 eggs. Before the experimental manipulation (i.e., increased testosterone and incubation regimen), egg mass did not differ among treatments (Generalized Linear Mixed Model (GLMM) with normal error distribution-treatment included as two-levels factors, and incubation batch and female identity as random factors, testosterone manipulation incubation temperature: $F_{1,\ 136.11} = 0.38$, $P = 0.54$).

Yolk testosterone manipulation

To simulate variation in maternal hormone deposition in the egg, we manipulated yolk testosterone concentration on the same day that incubation started. To synchronize the incubation period, eggs were collected within 4 days and were stored in an expanded polystyrene box in a dry place and away from sun exposure until testosterone and temperature manipulation was done. Eighty-two eggs were injected with 25 ng of testosterone (Sigma–Aldrich Chemie, Steinheim, Germany) diluted in 10 µl sesame oil (Santa Cruz Biotechnology, Santa Cruz, USA; i.e., increase testosterone eggs). Seventy-nine eggs were injected only with 10 µl sesame oil (i.e., control eggs). The amount of testosterone injected was within the natural range of variation for the species (4-30 ng/g of yolk; Hackl et al. 2003, Hayward et al. 2005, Okuliarova et al. 2007). During the egg injections, eggs were placed with the pointed end facing up on a LED lamp to visualize the yolk, the injection area was cleaned with 75% ethanol. Once the yolk migrated up to the egg-pointed end, the needle (30G x 13 mm) was inserted into the yolk and the solution was slowly injected, and the injection site was sealed with surgical glue. One egg per female was assigned, sequentially, to each treatment (i.e., testosterone or oil) and each incubation pattern (see below).

Incubation and yolk biopsy

To simulate cooling of the nest during incubation, increased testosterone and control eggs were sequentially assigned to one of two incubation treatments. Forty-one testosterone and thirty-nine oil-treated eggs were incubated at 38° C (± 0.56), using two cycles of 690 min in which the incubator (Grupo Morelos M-1, Morelos, Mexico) was set at 38° C, and then turned off during 30 min, reaching a minimum temperature of 35.9° C on average (range: 34.2 – 37.8° C). Forty-one increased testosterone and forty control eggs were incubated at 36° C (± 0.90), we used six cycles of 210 min in which the incubator was set at 36.9° C on average, then the thermostat was turned off for 30 min and the temperature dropped up to 34.7°C in average (range: 36.9° - 32.9°C). In both conditions, the humidity was set constant at 75 ± 1 % during the incubation period and at 83 ± 1 % three days before the estimated hatching date to facilitate the shell breaking and the chick hatching. Egg turning was performed automatically every hour (Oliveira et al. 2020). On day four of incubation, a yolk biopsy of 80 µL (less than 5% of the total yolk volume) was taken from all eggs to quantify hormone concentration; at this stage gonadal maturation has not been reaching, which allow us to analyze maternally derived hormones, avoiding the confounding effect of endogenous embryonic androgen production (Scheib et al. 1985, Carere and Balthazart 2007). Biopsies were performed following a protocol described elsewhere (see Hernández et al. 2022). Yolk samples were diluted in 100 µL deionized water and frozen at -80° C until hormone analyses. After the biopsy, eggs continued with the assigned incubation treatment until hatching.

Embryonic development

To assess the potential participation of the embryo in egg hormone metabolization, we evaluated the presence/absence of embryo development in the eggs that were previously injected and incubated, as the second step in our experiment. The presence/absence of the embryo was determined on day 4 of incubation, during the biopsy, through observation of the vascularization of the egg or the beating heart. Additionally, to confirm the determination of the presence/absence of an embryo on day 4 of incubation, we opened all eggs that did not hatch 3 (± 1.21) days after the estimated hatching day. Eggs that did not contain any sign of embryo

development were considered as infertile (i.e., absence of embryo development). Incubation temperature influenced the probability of finding an embryo present in the egg, those incubated at 38° C showed a lower probability of developing an embryo than eggs incubated at 36° C (Incubation temperature (Mean $\pm$ SD): 36° C = 0.72 $\pm$ 0.45; 38° C = 0.54 $\pm$ 0.50 Table S1, Figure S1) which could bias, at a certain degree, the results found or our ability to assess the predictions made here.

Hormone quantification

Steroid hormones in yolk samples were extracted twice by adding 1 mL of ether to 180 µL of diluted egg yolk (see incubation and yolk biopsy section), once evaporated, 1 mL methanol was added and left to sit until evaporated (López-García et al. 2013). Steroid hormone extracts used to quantify testosterone and estradiol were re-suspended by adding 200 µL phosphate-buffered saline (PBS), and an independent sample extract was resuspended with 300 µL PBS to quantify 5α-dihydrotestosterone. We quantified steroid hormone concentrations using ELISA Kits (DRG International, NJ, USA) by duplicate following the manufacturer protocol. Results are expressed as pg of hormone/mL. Intra-plate variation for testosterone was 3.67 % for 5α-dihydrotestosterone 3.55 %, and estradiol 2.85 %. Inter-plate variation was 6.66 %, 11.99 %, and 6.57 %, for testosterone, 5α-dihydrotestosterone, and estradiol respectively.

Statistical analyses

Before to statistical analyses, hormonal concentrations were log-transformed to meet normality assumptions. To assess the effect of the temperature on yolk hormone content, we analyzed testosterone, 5α-dihydrotestosterone, and estradiol titers as response variables by fitting independent GLMMs with normal error distribution (test included Shapiro-Wilk and visual inspection of model residual plotted against expected values). All models included incubation batch and female identity as random factors, yolk testosterone manipulation (i.e., increased testosterone or control injection), and incubation temperature (i.e., 36° C or 38° C) as fixed factors, and the interaction between these two main terms. Moreover, to explore the potential participation of embryos on testosterone metabolism, a GLMM including mother identity and incubation batch as random factors, yolk testosterone manipulation, incubation temperature, and

embryo presence/absence as fixed factors, and the interactions of embryo presence/absence with the other two factors (i.e., two two-way interactions and one three-way interaction) was adjusted. All analyses were conducted in R version 4. 1. 0 (R Development Core Team 2018). Final models were obtained by stepwise backward deletion of non-significant terms ($P > 0.05$). The sample sizes differ slightly among analyses due to missing data.

RESULTS

Influence of increased yolk testosterone and incubation temperature on testosterone metabolism
After four days of incubation, increased testosterone eggs had higher titers of testosterone compared to control eggs (Table 1). However, levels of yolk testosterone were not associated with incubation temperature or the interaction between testosterone manipulation and incubation temperature (Table 1, Figure 2a). Interestingly, 5α-dihydrotestosterone concentration in the yolk was independently influenced by both treatments, incubation temperature ($\beta = 0.17 \pm 0.05$, $F_{1,75.97} = 10.32$, $P = 0.002$) and yolk testosterone manipulation ($\beta = -0.12 \pm 0.05$, $F_{1,76.18} = 5.58$, $P = 0.02$) (Figure 2b), while the interaction between these two factors was not significant (Table 1). Levels of yolk estradiol were not associated with testosterone treatment, incubation temperature, or their interaction between them (Table 1, Figure 2c).

Influence of embryo development on yolk testosterone metabolism
We found no evidence that the presence of a developing embryo influences titers of testosterone, 5α-dihydrotestosterone, and estradiol in the yolk (Suppl. Mat. Table S2). The inclusion of embryo presence/absence in the models did not qualitatively modify the results described above (Suppl. Mat. Table S2).

DISCUSSION

In this study, we tested the hypothesis that besides maternal testosterone deposition in the yolk, incubation temperature may mediate the extent of embryo exposure to maternal androgens during the first days of development, through the effect of temperature on the metabolism of testosterone. We found that Japanese quail eggs that received a higher deposition of testosterone

and those incubated at 36° C increased 5α-dihydrotestosterone concentration in egg yolk, while no effects of temperature on titers of testosterone and estrogens were detected. Additionally, we found no evidence that the presence of the embryo may play a role in metabolizing egg yolk testosterone from maternal origin.

Lower incubation temperature was predicted to favor testosterone transformation to 5α-dihydrotestosterone while incubation temperature at 38° C was expected to trigger the conversion of testosterone into estradiol by enzymatic activity (Jeyasuria and Place 1998). Accordingly, we found that incubation temperature at 36° C as well as greater deposition of testosterone in the egg promoted higher yolk 5α-dihydrotestosterone titers, presumably as a result of the activity of the enzyme 5α-reductase. Studies in reptiles that evaluated the effect of incubation temperature on sex determination, through the modification of egg hormone titers, indicate that temperature modifies pH levels inside the egg affecting the expression of 5α-reductase, and consequently, the metabolization of testosterone into 5α-dihydrotestosterone (Jeyasuria and Place 1998). 5α-dihydrotestosterone is the most biologically potent androgen (Langlois et al. 2010) and its deposition in the yolk can entail phenotypic consequences, such as faster embryonic development, lower growth rate during the nestling period (Schwabl et al. 2007), and more exploratory behavior (Hegyi and Schwabl 2010). Our results show that incubation temperature can act as a maternal mechanism by which the phenotype of the offspring may be modified, similar to the findings of previous studies (DuRant et al. 2013, Hepp et al. 2015, Bertin et al. 2018, Wada et al. 2018). This result also suggests that environmental stimuli that trigger changes in hormone deposition into the eggs can affect offspring phenotype also via hormone transformation in other metabolites due to changes in incubation behavior that influence incubation temperature. We expected yolk testosterone levels in eggs with experimental androgen increase to decrease due to potential conversion into other compounds, however it did not occur. An explanation is that hormone manipulation increased testosterone concentration to levels that could be metabolized, but due to high levels of substrate (i.e., yolk testosterone levels) it is possible that despite the fact that this androgen could be transformed as a consequence of the incubation temperature, a reduction is not observed and testosterone will still be available.

Contrary to predictions, estradiol levels were not affected by incubation temperature. This may result if there is not enough aromatase transference from the mother into the egg yolk to allow testosterone metabolization during the first days of incubation, when aromatase from embryo origin is not yet produced (Egbert et al. 2013). Alternatively, incubation temperature could inhibit the action of this enzyme of maternal origin in the egg yolk; indeed, warmer incubation temperature can down-regulate aromatase (Pieau and Dorizzi 2004). A possible mechanism by which warmer temperatures may down-regulate aromatase is DNA methylation of this enzyme since incubation temperature has been found to increase the percentage of methylation of specific regions of the aromatase promotor which can suppress the gene expression and hence enzyme activity in reptiles and fishes with temperature-dependent sex determination (Navarro-Martín et al. 2011, Matsumoto et al. 2013). Nevertheless, the underlaying mechanisms by which incubation temperature affects enzymatic activity have been little explored, unraveling these mechanisms will advance our understanding of how maternal effects influence the offspring phenotype. Our results suggest that in Japanese quails, hormone-mediated maternal effects in the egg exert their action through androgens (testosterone and testosterone transformed into 5α-dihydrotestosterone), instead of testosterone conversion to estradiol.

Finally, we did not find any evidence of an active role by the embryo in metabolizing maternally derived testosterone in the egg yolk during the first four days of incubation. In our study, the presence of an embryo in the egg and its interaction with the increased testosterone or incubation temperature treatments were not associated with titers of testosterone, 5α-dihydrotestosterone, and estradiol. In the zebra finch, experimental evidence indicates that embryos start up taking or metabolizing yolk androgens between days 3 and 5 of incubation, as there was a decline in testosterone and 5α-dihydrotestosterone by day 5 of incubation and with increased size of the embryo (Gilbert et al. 2007). Differences in the stage of embryo development at the time of sampling and life history traits such as the velocity of embryo development between Zebra finches (altricial; Wada et al. 2015) and Japanese quails (precocial; Huss et al. 2008) may explain differences in metabolization of maternally derived androgens between these species. However, recent evidence in birds seems to suggest that embryos can metabolize or use maternally derived hormones in the first days of incubation (Vassallo et al.

2014, Kumar et al. 2019a, Campbell et al. 2020, Ruuskanen et al. 2022), but we were not able to detect the role of embryo due to the possible inability to determine with certainty the presence/absence of the embryo in the egg (associated with very early death) or due to lower statistical power.

Empirical evidence suggests that exposure to steroid hormones from maternal origin during early development has consequences on offspring phenotype (Podmokła et al. 2018). However, embryonic participation as modulator of the impact of these maternal hormones has received less attention. A potential role played by embryo may represent a source of prenatal parent-offspring conflict (Moore and Johnston 2008, Tobler and Smith 2010). Further experimental studies are needed to better understand the potential role of the embryo at metabolizing maternally derived testosterone in the Japanese quails. Some possibilities are the use of radio-labeled hormones and/or measuring hormone concentration during different embryonic stages throughout the incubation period.

In conclusion, we experimentally show that incubation temperature at 36° C increases embryo exposure to 5α-dihydrotestosterone during the first 4 days of incubation. This result suggests that incubation temperature, a condition of the developmental environment of the chick under the control of the mother, may constitute an additional pathway for hormone-mediated maternal effects beyond the well-studied deposition of maternal hormones in the egg. Most studies of maternal effects focus on the impacts of yolk hormones of maternal origin on the progeny phenotype, but incubation temperature is crucial for offspring development, hence female incubation behavior may be a maternal effect by which the exposure of embryos to testosterone may be modulated. Additionally, our manipulation did not find evidence for the role of the embryo in testosterone metabolism, however, further studies are needed to confirm if embryo is a passive receptor or it can modulate its exposure to maternal steroids. Our results open interesting avenues for future research aiming at understanding the fate of maternally derived hormones in the egg and the role played by female behavior during incubation on them. If variation in incubation temperature modifies testosterone metabolism, incubation temperatures may act as an important pathway for shaping adaptive offspring phenotype.

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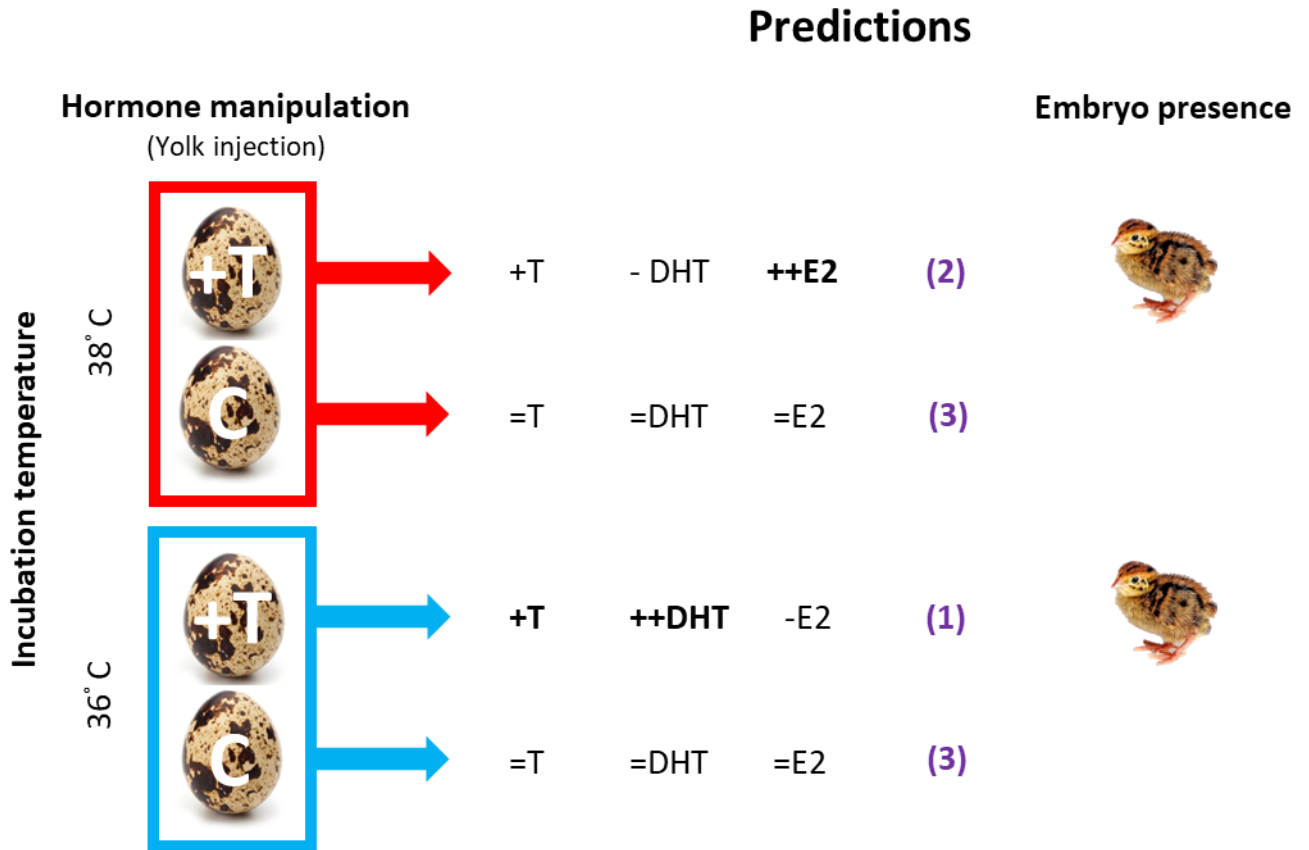
Figure 1. Predictions of the effects of incubation temperature increased testosterone in the egg and embryo presence/absence on the metabolism of testosterone in the yolk. +T: increased testosterone eggs; C: control eggs injected with oil; T: Testosterone, DHT: 5α-dihydrotestosterone, E2: Estradiol. Chick's imagen represents expected predictions about the role of embryo presence on metabolism of testosterone. Number in parenthesis correspond to predictions described in the text. +: High, ++: Higher, -: Lower, =: No differences. Number of eggs in each group (embryo presence/absence) quantified for T, E2, and DHT respectively: 38IT (19/22) = 20, 19, 21; 38C (23/14/ 2 NA) = 18, 17, 21; 36IT (28/13) = 22, 21, 19; 36C (30/10) = 19, 19, 20.
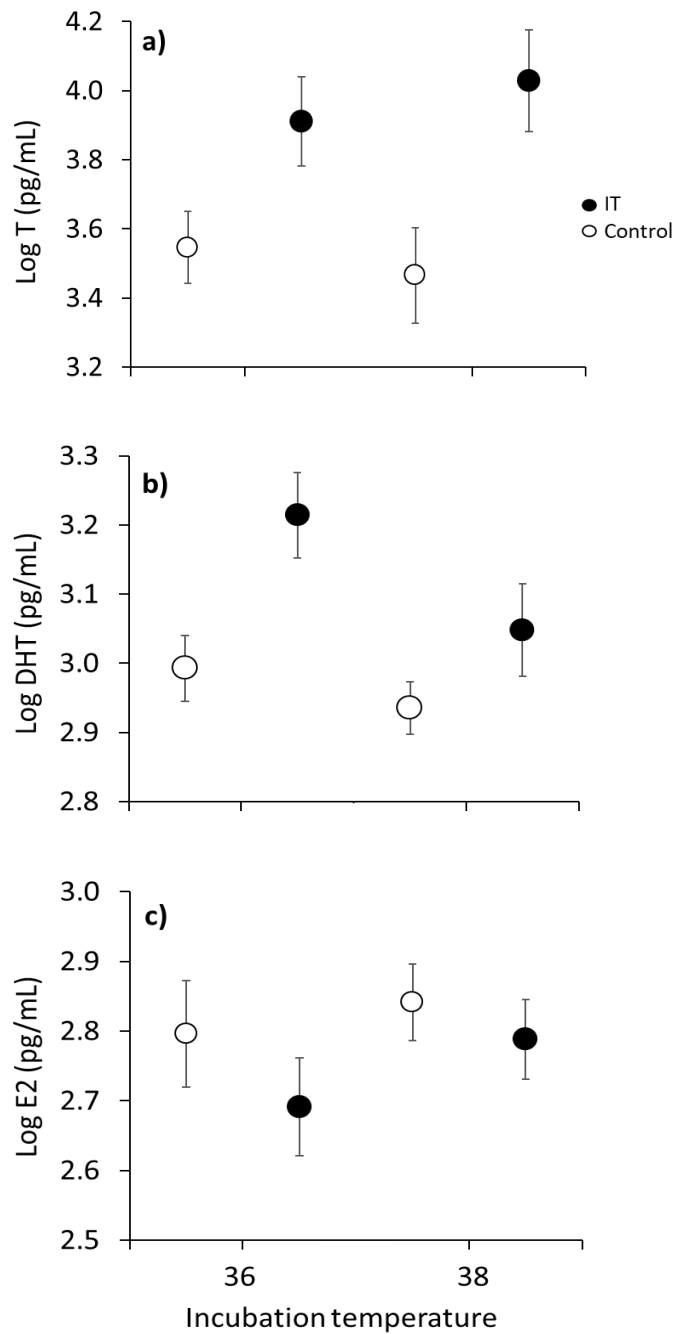
Figure 2. Effects of incubation temperature and increased testosterone treatment on the metabolism of testosterone in the yolk. Titers of a) testosterone (T), b) 5α-dihydrotestosterone (DHT), and c) estradiol (E2) of increase testosterone (IT) and control eggs incubated at 36° C and 38° C temperatures. The figures show mean ± SE.

Table 1. Effect of incubation temperature and increased testosterone in the egg yolk on testosterone metabolism. R^2-conditional of saturated model. IT: increase testosterone eggs.

	Mean ± SE (pg/mL)	Estimate ± SE	Df	*F*	*P*	R^2-cond
TESTOSTERONE						
Yolk testosterone manipulation	IT eggs: 19811.82 ± 3911.79 Control eggs: 5524.90 ±927.99	**0.52 ± 0.13**	**72.83**	**16.76**	**0.0001**	
Temperature	36° C: 11397.72 ± 2613.08 38° C: 14979.24 ± 3777.34	0.0006 ± 0.13	69.53	0.00	0.99	
Temperature × Yolk testosterone manipulation		0.17 ± 0.26	72.63	0.43	0.52	0.27
DHT						
Yolk testosterone manipulation	IT eggs: 1665.97 ± 181.84 Control eggs: 1015.75 ±75.90	**0.17 ± 0.05**	**75.97**	**10.32**	**0.002**	
Temperature	36° C: 1530.86 ± 173.79 38° C: 1156.68 ± 113.03	**-0.12 ± 0.05**	**76.18**	**5.58**	**0.02**	
Temperature × Yolk testosterone manipulation		-0.11 ± 0.10	75.04	1.09	0.30	0.28
ESTRADIOL						
Yolk testosterone manipulation	IT eggs: 663.90 ± 63.28 Control eggs: 799.48 ± 85.61	-0.07 ± 0.06	69.69	1.15	0.29	
Temperature	36° C: 716.86 ± 85.88 38° C: 740.64 ± 58.50	0.06 ± 0.06	65.04	0.94	0.34	
Temperature × Yolk testosterone manipulation		0.06 ± 0.13	66.56	0.23	0.63	0.18
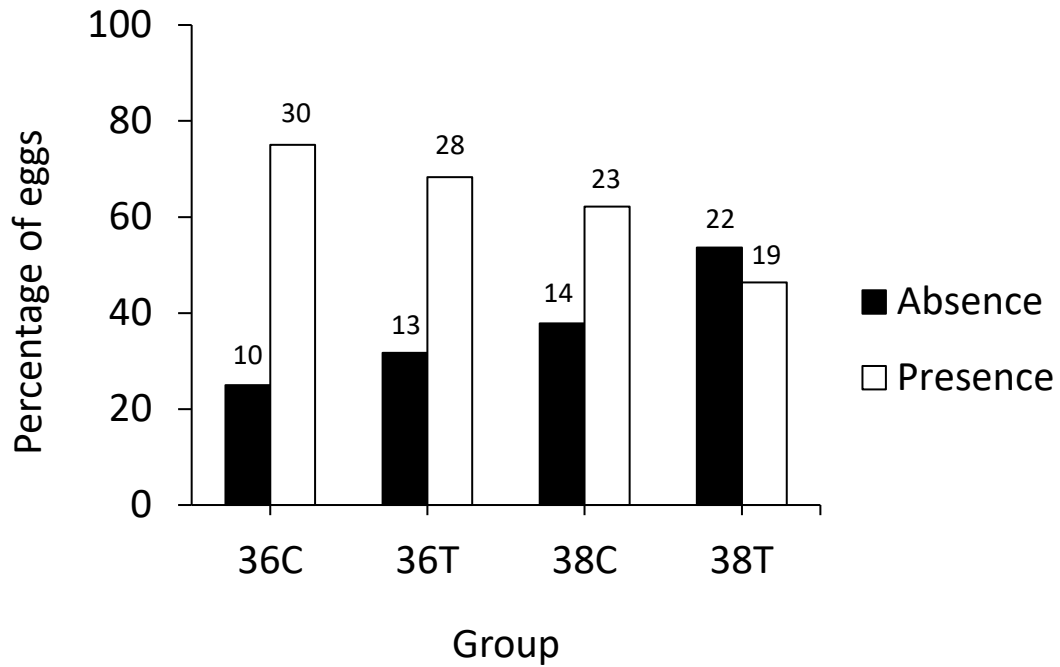
Figure S1. Percentage of eggs in each treatment in which the embryo presence /absence was recorded (36C-T: Increase testosterone and control eggs, respectively, incubated at 36° C, 38C-T: Increase testosterone and control eggs, respectively, incubated at 38° C). Numbers above the bars indicate sample size. In 38C, two eggs were can not examined after hatching date.

TABLES

Table S1. Probability of eggs to have an embryo at day four of incubation in relation to yolk testosterone and incubation temperature manipulation. GLMM with binomial error distribution, model included testosterone and incubation treatments and their interaction as main effects, and incubation batch as random factor; model simplification was achieved by deletion of non-significant terms by stepwise backward.

	Estimate ± SE	df	*F*	*P*
Yolk testosterone manipulation	-0.50 ± 0.34	155	-1.48	0.14
Temperature	**-0.77 ± 0.34**	**156**	**-2.30**	**0.02**
Temperature × Yolk testosterone manipulation	-0.31 ± 0.68	154	-0.46	0.65

Table S2. Effect of incubation temperature and increased testosterone on testosterone metabolism in eggs with and without an embryo. R^2-conditional of saturated model.

	Estimate ± SE	df	*F*	*P*	R^2-cond
TESTOSTERONE					
Yolk testosterone manipulation	**0.52 ± 0.13**	**72.83**	**16.76**	**0.0001**	
Temperature	0.0006 ± 0.13	69.53	0.00	0.99	
Embryo presence/absence	-0.03 ± 0.15	73.67	0.05	0.83	
Temperature × Yolk testosterone manipulation	0.22 ± 0.26	72.63	0.72	0.40	
Temperature × Embryo presence/absence	-0.08 ± 0.28	68.50	0.08	0.79	
Yolk testosterone manipulation × Embryo presence/absence	-0.06 ± 0.29	66.69	0.05	0.83	
Temperature × Yolk testosterone manipulation × Embryo presence/absence	0.91 ± 0.58	68.59	2.48	0.12	0.32
DHT					
Yolk testosterone manipulation	**0.17 ± 0.05**	**75.97**	**10.32**	**0.002**	
Temperature	**-0.12 ± 0.05**	**76.18**	**5.58**	**0.02**	
Embryo presence/absence	-0.01 ± 0.06	74.45	0.02	0.88	
Temperature × Yolk testosterone manipulation	-0.13 ± 0.10	73.03	1.49	0.23	
Temperature × Embryo presence/absence	0.08 ± 0.11	72.56	0.55	0.46	
Yolk testosterone manipulation × Embryo presence/absence	0.07 ± 0.11	71.85	0.39	0.54	

Temperature × Yolk testosterone manipulation × Embryo presence/absence	-0.16 ± 0.23	70.12	0.51	0.48	0.28
ESTRADIOL					
Yolk testosterone manipulation	-0.05 ± 0.07	66.71	0.66	0.42	
Temperature	0.10 ± 0.07	64.62	2.40	0.13	
Embryo presence/absence	0.10 ± 0.07	72.99	2.44	0.12	
Yolk testosterone manipulation × Embryo development	-0.16 ± 0.13	65.03	1.39	0.24	
Temperature × Embryo presence/absence	-0.06 ± 0.14	63.80	0.20	0.66	
Temperature × Yolk testosterone manipulation	0.08 ± 0.14	66.45	0.36	0.55	
Temperature × Yolk testosterone manipulation × Embryo presence/absence	0.03 ± 0.29	64.85	0.01	0.92	0.24

DISCUSIÓN GENERAL

En las aves, factores sociales y ambientales a los que expuestas las hembras pueden propiciar un aumento en la deposición de testosterona en la yema, por ejemplo, en ambientes con altas interacciones agresivas o competitivas (Bentz et al. 2016), o una mayor concentración de corticosterona en el huevo cuando la disponibilidad de recursos es baja o ante eventos estresantes (Love et al. 2005). Estas respuestas hormonales de la hembra pueden afectar positiva o negativamente el fenotipo de las crías a corto y largo plazo (Tabla 1).

Durante tres décadas, los efectos maternos, *i. e.* la influencia del fenotipo materno o de las condiciones ambientales experimentadas por las madres en la descendencia (Mousseau y Fox 1998), han sido explorados, principalmente, evaluando las variaciones en la concentración de hormonas depositadas en el huevo y sus efectos en el fenotipo de las crías. Sin embargo, poco se ha investigado sobre mecanismos maternos adicionales que podrían ocurrir posteriormente a la deposición de estas señales químicas, *e. g.* modificaciones en los patrones de incubación o el destino de las hormonas durante el desarrollo embrionario de la progenie. Estas indagaciones son necesarias para determinar si estas sustancias impactan directamente en la cría o se metabolizan a lo largo del período de incubación. Además, recientemente se ha planteado la posibilidad de que la descendencia no actúa como un receptor pasivo de las hormonas depositadas por la madre, sino que medie su exposición a estos mensajeros químicos (Vassallo et al. 2019, Campbell et al. 2020, Paiz et al. 2020).

En esta tesis investigamos si las hembras del azulejo de garganta azul responden a las interacciones competitivas modificando la concentración de testosterona depositada en la yema, y si la competencia materna promueve cambios a corto plazo en el fenotipo de las crías. Los resultados del capítulo 1 muestran que el ambiente competitivo al que las hembras fueron expuestas no promueve una mayor deposición de testosterona en el huevo; sin embargo, este ambiente sí repercute en el crecimiento, comportamiento y supervivencia temprana (marginalmente) de la descendencia. Estas variaciones en las trayectorias de desarrollo podrían indicar alteraciones en el cuidado parental (*e. g.* la tasa de alimentación), no obstante, en este trabajo no se evaluó la atención de los padres en el nido. Si bien estudios previos reportaron la

asignación de andrógenos en la yema de los azulejos en relación con la disponibilidad de sitios de anidación (Duckworth et al. 2015), las discrepancias en los resultados podrían derivar de las diferencias en las condiciones ecológicas entre poblaciones de la misma especie, sugiriendo que los efectos maternos pueden ser contexto-dependientes (Marshall y Uller 2007). Bajo este escenario, es probable que variaciones en las estrategias de dispersión y reproductivas entre las poblaciones influyan en la respuesta de las hembras ante los eventos competitivos y, por ende, en los efectos maternos que podrían dispararse ya que los azulejos del Centro de México no migran latitudinalmente y presentan eventos reproductivos más breves (Parque Nacional La Malinche: Abril-Junio con un único evento reproductivo al año *vs.* Montana: Abril–Agosto con dos intentos de anidación; Potticary y Duckworth 2018, 2020, Porras-Reyes et al. 2021). Sería interesante que futuros estudios evalúen si las crías de hembras expuestas a altos niveles de competencia por los sitios de anidación presentan el fenotipo dispersivo y agresivo reportado en otras poblaciones pese a que en esta población los embriones parecen no estar expuestos a altos niveles de testosterona.

En el capítulo 2 se puso a prueba la idea de que los cambios en las trayectorias de crecimiento de las crías de azulejo, derivados del ambiente competitivo al que estuvo expuesta la madre, podrían estar mediados por la acción del incremento en los niveles de corticosterona y podrían emerger como costos de un posible aumento en el daño oxidante de la progenie. Los resultados mostraron que los niveles de corticosterona y daño en lípidos en sangre de los polluelos a los 3 días de edad (*i. e.* medición más cercana a la eclosión) no difirieron entre las crías de las hembras expuestas a altos y bajos niveles de competencia durante la construcción del nido. Sin embargo, ambos parámetros fisiológicos actuaron como un mecanismo vinculante entre el ambiente prenatal materno y el crecimiento de la descendencia. Las crías macho controles con una mayor concentración de corticosterona circulante desarrollaron tarsos más largos a la misma edad que las crías macho experimentales. Por el contrario, las crías controles mostraron tarsos más cortos cuando tenían niveles altos de peroxidación de lípidos, mientras que en las crías de hembras expuestas a los eventos competitivos no se encontraron asociaciones entre la talla y los parámetros fisiológicos analizados. Estos resultados son de interés porque las asociaciones encontradas no habían sido exploradas en esta especie previamente, siendo que durante la vida temprana se desarrollan las funciones vitales del organismo que definirán su

éxito en una población. Asimismo, los efectos de los niveles de corticosterona y daño oxidante en lípidos sobre el desarrollo del esqueleto podrían estar asociados a diferencias sexuales en mecanismos fisiológicos involucrados.

Finalmente, el capítulo 3 de esta tesis evaluó si la temperatura de incubación influye en el metabolismo de la testosterona materna depositada en el huevo y si el embrión podría estar modulando su exposición a la testosterona depositada por la madre, metabolizando esta hormona. La codorniz japonesa (*Coturnix japonica*) resulta un objeto de estudio óptimo para la evaluación de estas ideas debido a la facilidad de la incubación artificial de los huevos y al corto período de incubación (16 días aproximadamente; Huss et al. 2008). Se encontró que temperaturas de incubación bajas (36° C) promueven niveles más altos de 5α-dihidrotestosterona. Por otro lado, no hay evidencia que apoye la idea de que el embrión participa en la metabolización de la testosterona a 5α-dihidrotestosterona o estradiol. Los resultados sugieren que la temperatura de incubación actúa como un mecanismo por el cual la madre podría influir en el fenotipo de la progenie, debido a su impacto sobre el metabolismo de la testosterona, mientras que durante los primeros días de incubación (período evaluado en este estudio) el embrión no parece modular su propia exposición a la testosterona materna. Por lo tanto, estos datos plantean un posible escenario, muy poco estudiado hasta ahora, en el que diferentes efectos maternos, es decir la cantidad de testosterona depositada en el huevo y la conducta de incubación, podrían interactuar abriendo la posibilidad de que, aún después de que los huevos son puestos, las hembras sigan influyendo en el fenotipo de sus crías.

Perspectivas

Los resultados de esta tesis en conjunto plantean varias preguntas para investigaciones futuras:

El supuesto de que las hembras responden a los eventos competitivos durante la temporada reproductiva incrementando la deposición de testosterona en el huevo no fue apoyado por los resultados que se reportan en el capítulo 1, por lo que estudios futuros deberían considerar la influencia de las diferencias en las condiciones ecológicas de las poblaciones (*e. g.* ambientales, sociales y reproductivas) en la ocurrencia de los efectos maternos mediados por hormonas como respuesta a la competencia a recursos claves.

Igualmente, es de interés evaluar el vínculo entre los efectos a corto y largo plazo que tiene la variación en factores ecológicos y sociales, ya que si los mecanismos subyacentes a la competencia materna difirieron de aquellos descritos previamente (*i. e.* diferencias en la deposición de andrógenos en yema), las consecuencias en términos de adecuación también podrían ser distintas.

Por otro lado, resulta de interés evaluar las implicaciones de las variaciones en los patrones de incubación como mecanismos adicionales a la modificación de hormonas en la yema como un vínculo entre el ambiente experimentado por la hembra y los atributos de su descendencia, ya que variaciones en la temperatura de incubación, por un lado, pueden alterar directamente el fenotipo de los polluelos y, por otro lado, transformar las hormonas previamente depositadas en el huevo durante su producción. Igualmente, podría ser relevante investigar si existe una ventana temporal sensible a las señales hormonales y cómo la exposición y transformación de las hormonas maternas durante el desarrollo embrionario modelarían un fenotipo que le confieran ventajas adaptativas.

Finalmente, la evaluación de los mecanismos potenciales a través de los cuales los esteroides de origen materno son metabolizados podrían asociarse con un conflicto padre-hijo donde la cría desarrolla una estrategia de defensa contra la influencia hormonal materna.

Conclusiones

Aunque los efectos maternos mediados por hormonas han sido ampliamente estudiados en las últimas décadas, la función adaptativa tanto para la madre como para la progenie ha intrigado a los investigadores a lo largo de los años. Esta tesis sugiere que la influencia de las interacciones sociales experimentadas por la madre y los cambios en el cuidado parental de la descendencia podrían jugar un rol determinante sobre las trayectorias de desarrollo de las crías a corto y largo plazo. Sin embargo, el mecanismo por el cual actúan los efectos maternos, y consecuentemente la función adaptativa, podría depender del contexto ecológico particular. Asimismo, el efecto de las condiciones ambientales puede acarrear repercusiones dependientes del sexo y condición del individuo y ocurrir en ventanas sensibles del desarrollo. Por otro lado, los datos presentados en el capítulo 3 sugieren un potencial mecanismo que interviene en los efectos de las hormonas

depositadas por las madres en el huevo, ya que se plantea otro elemento, *i. e.* la temperatura de incubación, como un efecto materno adicional que puede participar en el ajuste del fenotipo de la progenie.

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APÉNDICES

CAPÍTULO 1: POST THE BOU BLOG
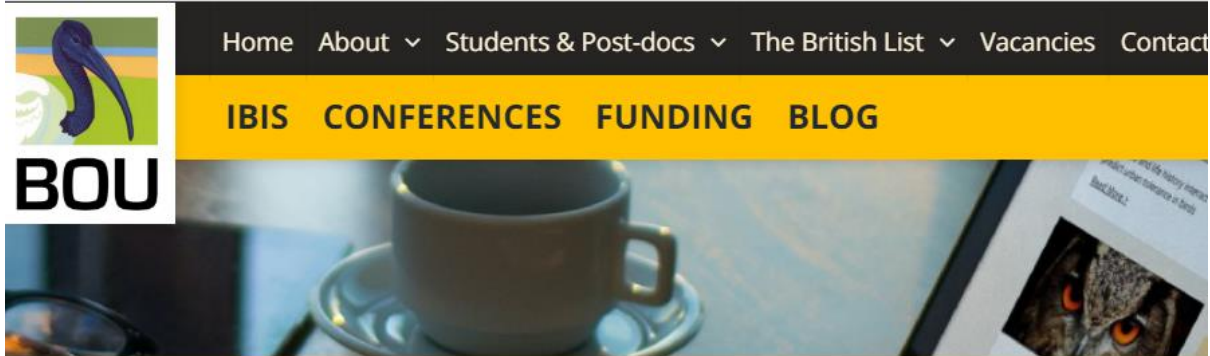
Home > Maternal competitive events influence offspring phenotype

5 Sep 2022

Posted in: avian biology, Breeding, breeding biology, cavity nesting, competition, early-career researcher, ecology, ECR, eggs, experiment, IBIS, nest monitoring, nest-site limitation, passerines, phenotypic variation

Maternal competitive events influence offspring phenotype

Compete with me and my progeny will grow faster

América Hernández
Tlaxcala Center for Behavioral Biology, Mexico

LINKED PAPER
Competitive events during nest building influence offspring growth and behaviour, but not testosterone deposition in the egg yolk in Western Bluebirds (*Sialia mexicana*). Hernández, A., Hernández-Martínez, A. Nicolás, L., Torres, R. & Montoya, B. 2022 *Ibis*. doi: 10.1111/ibi.13098 VIEW

Western Bluebirds (*Sialia mexicana*) are small passerines that breed from South Canada to Central Mexico. This species breeds on cavities (natural on trees or artificial) that they do not excavate by their own (i.e., they are secondary cavity nester). Western Bluebirds compete with other secondary cavity species for nest sites, and these competitive interactions can trigger changes in the amount of testosterone the mother transfer to the egg (i.e., a testosterone-mediated maternal effect), and these modifications in the egg content can have short- and long-term impacts on the progeny.

Figure 1 Male (on the top) and female (in the box entrance) Western Bluebird (*Sialia mexicana*).

For example, in northern populations of this species, female competitive interactions during early stages of egg formation promotes the development of dispersive and aggressive male offspring at sexual maturity, as a consequence of the increase in yolk testosterone level (Duckworth *et al*. 2015) and these offspring traits may allow them to colonize new breeding areas. However, competitive pressures for nest sites vary through the range of distribution of Western Bluebirds and this variation may influence female breeding strategies, the stimulus that trigger maternal effects, and the type of maternal effects displayed. The importance of ecological variation on maternal effects and the potential consequences on offspring phenotype deserve further investigation.

Figure 2 Western Bluebirds USFWS – Pacific Region CC BY-NC 2.0 Flickr.

In our study, we followed one of the southernmost populations of Western Bluebirds (La Malinche National Park, Tlaxcala, Mexico) and experimentally increased the level of competition for nest cavities during the nest-building period. To increase the perceived level of competition, we decreased the number of available nestboxes near the experimental nest and simulated territorial intrusions of the main competitor of Western Bluebirds in the population of study by displaying a 3D model and a playback of a House Wren (*Troglodytes aedon*) twice a week from nest construction to egg laying. In the control group, no boxes were removed and the simulated intrusions and playbacks were performed with a 3D model and vocalizations of a Red Warbler (*Cardellina rubra*), a ground nesting species that do not compete with Western Bluebirds. After clutch completion, we took an egg yolk sample to quantify testosterone concentration, and we recorded the offspring growth, behaviour (breathing rate and tonic immobility) and fledging survival.

We found that Western Bluebird females from this Southern population did not modify yolk testosterone levels in response to increased nest competition. However, the experimentally increased competition influenced the offspring phenotype. Chicks in the experimental group were lighter at the age of three days, then exhibited an accelerated growth rate in the first half of the nesting period, followed by a slower growth gain in the second one, and ended up with larger tarsus at age 15 days compared to chicks in the control group. Moreover, experimental chicks exhibited lower breathing rate and marginally lower fledging survival probability.

Unlike the northern populations where maternal competition for breeding sites influences offspring phenotype through changes in egg yolk testosterone deposition, in the southern population studied here, maternal competition affected offspring growth through mechanisms that do not involve changes in egg yolk testosterone. Parental behaviour post-hatching may be a candidate mechanism linking maternal competition to changes in offspring phenotype, if parents exposed to increased competition modify parental care influencing offspring growth, behaviour and survival (Siefferman & Burdick 2020). Taken together, our results suggest that ecological differences among populations may determine the type of maternal effects triggered, the key stimuli and its consequences on the offspring (Albers *et al*. 2017).

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Siefferman, L.M., & Burdick, C. 2020. Interspecific density influences the adaptive significance of provisioning coordination between breeding partners. *Frontiers in Ecology and Evolution* 8: 29 VIEW

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Colourful traits in female birds relate to individual condition, reproductive performance, and male mate preferences: A meta-analytic approach**

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Running head: **Female colorful traits: A meta-analysis**

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Colourful traits in female birds relate to individual condition, reproductive performance and male-mate preferences: a meta-analytic approach

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Colourful traits in females are suggested to have evolved and be maintained by sexual selection. Although several studies have evaluated this idea, support is still equivocal. Evidence has been compiled in reviews, and a handful of quantitative syntheses has explored cumulative support for the link between condition and specific colour traits in males and females. However, understanding the potential function of females' colourful traits in sexual communication has not been the primary focus of any of those previous studies. Here, using a meta-analytic approach, we find that evidence from empirical studies in birds supports the idea that colourful female ornaments are positively associated with residual mass and immune response, clutch size and male-mate preferences. Hence, colourful traits in female birds likely evolved and are maintained by sexual selection as condition-dependent signals.

1. Introduction

Although females of many species exhibit conspicuous traits, research on the evolution of extravagant characters through sexual selection has mainly focused on male ornaments [1]. Hypotheses explaining the occurrence of female ornamentation propose that female ornaments evolved because they share genetic architecture with male ornaments, confer privileges during the competition for ecological resources or are advantageous during sexual selection [2–5]. Comparative studies indicate that selective pressures favoured the evolution of female ornamentation, but whether those pressures arise mainly from natural selection, female–female competition or male-mate preferences is unclear [1,6,7]. There is growing evidence that male-mate choice has played a role in the evolution of female ornamental traits [5,8–12], but the overall strength of that evidence has seldom been systematically evaluated.

There are several (non-exclusive) scenarios where sexual selection is expected to favour the evolution of female ornaments, for instance, when the variation in females' reproductive value is large and is related to their ornamentation; when there are more females available than males can mate with and/or mating with one female reduces the opportunity to mate with another; and under reversed sex roles [11–15]. Female ornamentation is also expected in the context of mutual mate choice, where acquiring information from ornaments about the

potential direct or indirect benefits of mating with a particular partner may be beneficial to both sexes, such as in species with biparental care and serial monogamy [16–18]. However, male-mate choice has been shown to occur even in polygynous species, as evidenced by differential sperm transfer depending on a female's attractiveness [19–21].

Birds exhibit striking colours, and the role of male color-ation as a condition-dependent signal has been extensively studied in this taxon [22–24]. However, females have under-gone evolutionary change more frequently than males, and changes have often been towards more ornamented traits in females, suggesting that selection may have acted on male and female ornaments independently [25]. Hence, birds may be a good model for studying the evolution of female ornamentation. Coloured avian traits are mainly produced by carotenoids, melanin and tissue structures [23,26]. These three types of coloration depend on different underlying mech-anisms and therefore may communicate different information [25]. Carotenoid coloration depends on individuals' capacity to acquire, metabolize and store carotenoids from their diet [27] and have been positively linked to immune competence and oxidative balance ([28–39], but see [40]). Melanin-based colours are ubiquitous in birds; they rely on a synthesis path-way that releases cytotoxic compounds and requires metal cofactors that are functionally linked to other physiological functions [23]. Frequently, melanins have been associated with social status, but what type of condition-dependent infor-mation melanin-based colours may provide is less clear [41,42]. Finally, colours that result from the interaction between light and the arrangement of the nanostructure (i.e. array of melano-somes and keratin matrix) and microstructure of tissues (i.e. array of barbs or barbules) have received comparatively less attention in this context [43–46] but a recent meta-analysis found them to be correlated to residual body mass and immune function [47]. Whether these different colour displays are condition-dependent sexually selected signals used by males to evaluate females in a way that is analogous to female-mate choice is still under debate [18,48–52].

Here, using a meta-analytic approach, we evaluate the available evidence from published studies assessing the idea that conspicuously coloured traits, in female birds, have evolved through sexual selection as condition-dependent sig-nals. We expect female colour traits to be positively associated with the female condition, reproductive performance and male-mate preferences.

2. Methods

A systematic search of the literature was performed using the PRISMA method (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [53]. We looked for literature using the Google Scholar and Web of Science search engines, using the keywords: 'female bird ornament', 'female bird ornamenta-tion', 'female bird traits', 'female bird quality', 'female bird condition', 'female bird attractiveness', 'female bird colour', 'female bird color', 'female bird coloration', 'male bird choice' and 'male bird preference'. References within papers were also checked. The last search was conducted on 15 October 2020.

We excluded studies that were missing data on statistical values, did not provide independent estimates for each sex or did not specify the type of coloration analysed. We detected 83 studies in our search, 59 of which satisfied the inclusion criteria (electronic supplementary material, figure S1, [54]). Of the 47

total species (eight species were used in more than one study), 42 had a socially monogamous mating system, four were facultatively polygamous and one was polygamous. Carotenoid-dependent ornaments were present in 23 species, melanin-based in 18 and structural colorations in 11 (electronic supplementary material, table S1). In the studies included in the meta-analyses, coloration was originally estimated using the number and/or size of coloured structures/patches, spectrophotometry, digital image analysis (RGB or LBA) and visual rank scales (colour charts and visual rank score).

Using independent meta-analyses, we evaluated the evi-dence of an association of colourful female ornaments with the condition, reproductive performance and male preferences (see below). Studies assessing more than one of these associations were included in each meta-analysis that applied. Effect sizes for quality and reproductive success were analysed as Pearson's correlation coefficients (r). When this coefficient was not directly available from papers (i.e. different statistics were reported, such as F, t and χ^2), reported values were transformed to correlation coefficients following Rosenberg's formulae [55]. Pearson's coef-ficients were subsequently transformed to Fisher's z-values (Zr) for statistical analyses ([56], see equations in the electronic sup-plementary material). All effect sizes were weighted using the inverse of the sampling variance [57,58]. Effect sizes for male pre-ferences were reported as Hedges' g, calculated from Pearson's coefficients (reported statistics were transformed when necessary, see above) [56], and weighted using the g' variance ([56], see equations in the electronic supplementary material).

Three moderators were considered in the model evaluating the association between female colour and condition: condition proxy (residual body mass, immune response—humoral or cellular— and parasite load), ornament type (feathers or integuments) and coloration type (carotenoid-dependent, melanin-based or struc-tural). In the model evaluating the association between female colour and reproductive performance, we used the same ornament type and coloration type moderator variables but replaced the condition proxy with a reproductive performance proxy (laying date, clutch size or fledging success). When the clutch size and fledging success were assessed in the same study, only fledging success was considered. In the analyses exploring the relationship between female colour and male mating preferences, only four effect sizes were available for melanin-based and structural colours (one and three, respectively), so we fit this model including effect sizes only from studies evaluating carotenoid-dependent color-ation ($n = 11$). The ornament type (feathers or integuments) was included as a moderator.

In the case that two or more effect sizes were available from a single paper testing the same hypothesis and using the same ornament type and coloration (e.g. two carotenoid-dependent feather patches), those effect sizes were averaged to avoid over-representation. In studies that contained multiple effect sizes from different ornament or coloration types (e.g. one carotenoid-dependent feather patch and one melanin-based integument patch), we included each of the effect sizes separately and study identity as a random factor. We used Cochran's Q as a measure of effect size heterogeneity and the Q_E-test and Q_M-test to estimate whether moderators were associated with estimates of effect size. Publication bias was illus-trated using funnel plots [59] and assessed by the trim and fill method [60,61]. All analyses were conducted using the R package *metafor* [62] and the function *rma.mv* [62] in R v. 4.0.2 software [63].

Ornaments exhibited by different species may share evolution-ary history, generating phylogenetic non-independence. To account for this, a phylogeny for each analysis was obtained using a maxi-mum clade credibility consensus tree and a sample of 100 phylogenies downloaded from BirdTree (www.birdtree.org) [64] based on the Ericson *et al.* [65] backbone. The influence of phylo-genetic signal was assessed using two independent approaches (electronic supplementary material, table S2). First, we determined
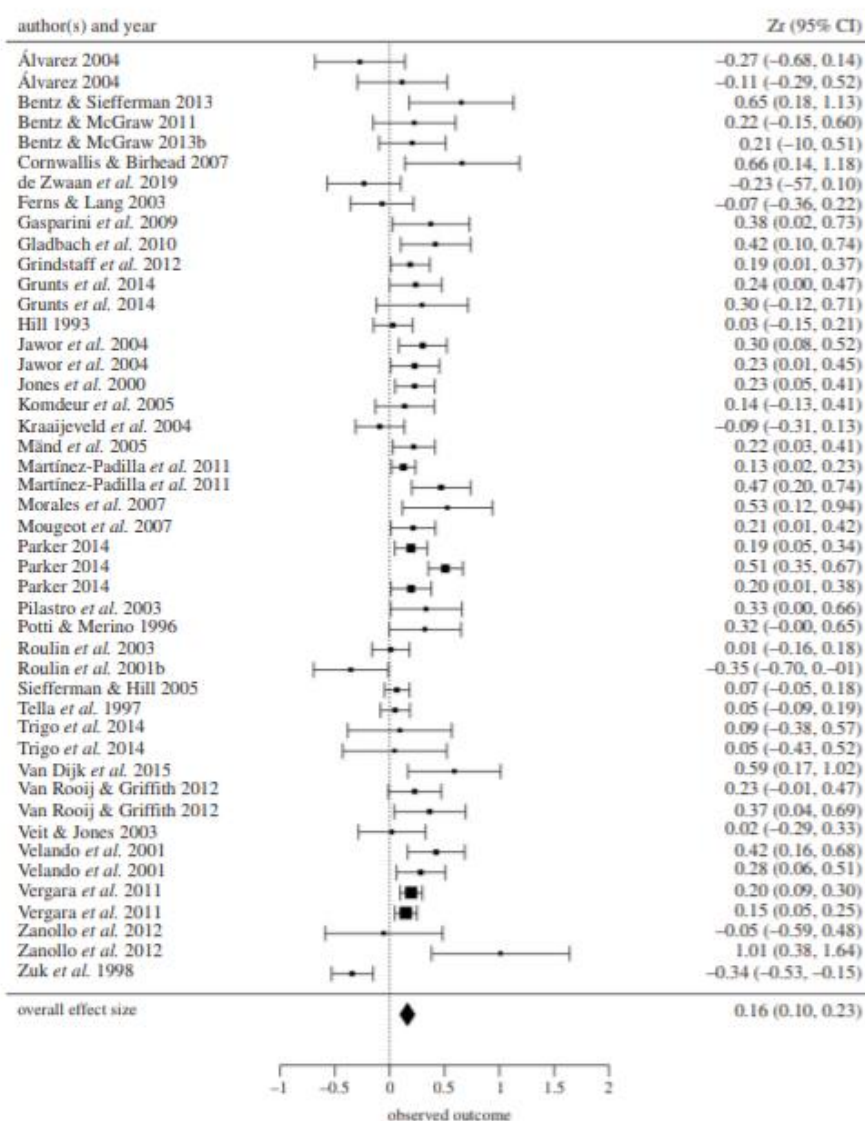
author(s) and year	Zr (95% CI)
Álvarez 2004	-0.27 (-0.68, 0.14)
Álvarez 2004	-0.11 (-0.29, 0.52)
Bentz & Siefferman 2013	0.65 (0.18, 1.13)
Bentz & McGraw 2011	0.22 (-0.15, 0.60)
Bentz & McGraw 2013b	0.21 (-10, 0.51)
Cornwallis & Birhead 2007	0.66 (0.14, 1.18)
de Zwaan et al. 2019	-0.23 (-57, 0.10)
Ferns & Lang 2003	-0.07 (-0.36, 0.22)
Gasparini et al. 2009	0.38 (0.02, 0.73)
Gladbach et al. 2010	0.42 (0.10, 0.74)
Grindstaff et al. 2012	0.19 (0.01, 0.37)
Grunts et al. 2014	0.24 (0.00, 0.47)
Grunts et al. 2014	0.30 (-0.12, 0.71)
Hill 1993	0.03 (-0.15, 0.21)
Jawor et al. 2004	0.30 (0.08, 0.52)
Jawor et al. 2004	0.23 (0.01, 0.45)
Jones et al. 2000	0.23 (0.05, 0.41)
Komdeur et al. 2005	0.14 (-0.13, 0.41)
Kraaijeveld et al. 2004	-0.09 (-0.31, 0.13)
Mänd et al. 2005	0.22 (0.03, 0.41)
Martínez-Padilla et al. 2011	0.13 (0.02, 0.23)
Martínez-Padilla et al. 2011	0.47 (0.20, 0.74)
Morales et al. 2007	0.53 (0.12, 0.94)
Mougeot et al. 2007	0.21 (0.01, 0.42)
Parker 2014	0.19 (0.05, 0.34)
Parker 2014	0.51 (0.35, 0.67)
Parker 2014	0.20 (0.01, 0.38)
Pilastro et al. 2003	0.33 (0.00, 0.66)
Potti & Merino 1996	0.32 (-0.00, 0.65)
Roulin et al. 2003	0.01 (-0.16, 0.18)
Roulin et al. 2001b	-0.35 (-0.70, 0.-01)
Siefferman & Hill 2005	0.07 (-0.05, 0.18)
Tella et al. 1997	0.05 (-0.09, 0.19)
Trigo et al. 2014	0.09 (-0.38, 0.57)
Trigo et al. 2014	0.05 (-0.43, 0.52)
Van Dijk et al. 2015	0.59 (0.17, 1.02)
Van Rooij & Griffith 2012	0.23 (-0.01, 0.47)
Van Rooij & Griffith 2012	0.37 (0.04, 0.69)
Veit & Jones 2003	0.02 (-0.29, 0.33)
Velando et al. 2001	0.42 (0.16, 0.68)
Velando et al. 2001	0.28 (0.06, 0.51)
Vergara et al. 2011	0.20 (0.09, 0.30)
Vergara et al. 2011	0.15 (0.05, 0.25)
Zanollo et al. 2012	-0.05 (-0.59, 0.48)
Zanollo et al. 2012	1.01 (0.38, 1.64)
Zuk et al. 1998	-0.34 (-0.53, -0.15)
overall effect size	0.16 (0.10, 0.23)

observed outcome

Figure 1. Association of colourful female traits with condition (effect sizes ± 95% confidence intervals).

the phylogenetic signal in model residuals as Pagel's λ [66] and Blomberg's Kappa (K) [67] using the *phylosig* function from the *phytools* package [68]. Second, we used phylogenetic generalized least-squares models to estimate Pagel's λ through maximum likelihood, by fitting the model as effect sizes approximately 1, using the R package *caper* [69]. Results obtained suggest that closely related species show similar relationships between female coloration and reproductive performance (electronic supplementary material, table S2) Here, we present the results of the analyses including phylogeny, following Nakagawa & Santos [70].

3. Results

Thirty-five studies (46 effect sizes) analysed supported a positive association between female colour and condition ($Zr = 0.16 \pm 0.03$, $p < 0.0001$; figure 1). Females' colourful

characters were positively associated with residual mass ($Zr = 0.16 \pm 0.04$, $p < 0.0001$) and immune response ($Zr = 0.31 \pm 0.10$, $p = 0.002$) but not parasite load (table 1a). Structural, carotenoid-dependent and (marginally) melanin-based coloration of plumage as well as integuments were related to the female condition (table 1a). Publication bias was found, suggesting eight missing studies supporting the null hypothesis (electronic supplementary material, figure S2). However, when the effect size considering missing studies was calculated equivalent results were obtained ($Zr = 0.13 \pm 0.03$, $p = 0.0003$). Finally, Q_E was significant, which suggests that there is still variance that is unaccounted for by the model (table 1a).

Twenty-nine studies (42 effect sizes) included in the analysis provided support for a positive association between female colour and reproductive performance ($Zr = 0.15 \pm 0.06$, $p = 0.01$; figure 2a). Particularly, female colour was positively associated

Table 1. Effect sizes (Zr) of moderator variables for the relationships of colourful female ornaments with condition (a) and reproductive performance (b). Overall effect sizes were obtained in the absence of moderator variables.

	Zr	s.e.	P	residual heterogeneity		moderator heterogeneity	
				Q_E	P	Q_M	P
(a) condition							
overall	0.16	0.03	<0.0001				
proxy				122.23	<0.0001	29.30	<0.0001
residual mass	0.16	0.04	<0.0001				
immune response	0.31	0.10	0.002				
parasite load	0.09	0.05	0.08				
ornament type				126.60	<0.0001	21.03	<0.0001
integument	0.18	0.05	0.0008				
plumage	0.15	0.04	<0.0001				
coloration type				124.27	<0.0001	25.05	<0.0001
carotenoid	0.18	0.05	<0.0001				
melanin	0.10	0.05	0.05				
structural	0.22	0.07	0.002				
(b) reproductive performance							
overall	0.15	0.06	0.01				
proxy				135.40	<0.0001	6.41	0.09
clutch size	0.28	0.14	0.04				
fledging success	0.13	0.07	0.07				
laying date	0.13	0.07	0.07				
ornament type				135.80	<0.0001	5.06	0.08
integument	0.31	0.14	0.03				
plumage	0.10	0.08	0.23				
coloration type				119.17	<0.0001	10.52	0.01
carotenoid	0.26	0.08	0.002				
melanin	0.08	0.07	0.26				
structural	0.15	0.08	0.07				

with clutch size ($Zr = 0.28 \pm 0.14$, $p = 0.04$). Coloration type explained heterogeneity among studies ($Q_M = 10.52$, $p = 0.01$), carotenoid colours were positively linked to reproductive performance ($Zr = 0.26 \pm 0.08$, $p = 0.002$). Publication bias was found, but the result was qualitatively equivalent even after accounting for the 11 missing studies, following the trim and fill method ($Zr = 0.08 \pm 0.04$, $p = 0.056$; electronic supplementary material, figure S3). Residual heterogeneity was found (table 1b).

Eleven experimental studies (11 effect sizes) analysed supported the idea that males prefer females with more colourful carotenoid-dependent traits ($g = 0.64 \pm 0.11$, $p < 0.0001$; figure 2b). Integument colour ($g = 0.60 \pm 0.17$, $p = 0.0004$) and plumage colour ($g = 0.71 \pm 0.21$, $p = 0.0009$) explained the heterogeneity among studies ($Q_M = 17.24$, $p = 0.0002$). Neither unaccounted variance ($Q_E = 16.36$, $p = 0.06$), nor publication bias was identified (electronic supplementary material, figure S4).

4. Discussion

Using a meta-analytic approach, we found support for an association between colourful female traits and individual condition (measured as residual mass and immune response), clutch size and male-mate preferences. Hence, the expression of colourful characters in female birds is likely to be maintained by male preferences due to its probable role as a signal of the female condition and reproductive potential.

The evidence we analysed supported a positive association between colour and condition (figure 1). Thus, our results suggest that, like in males of a great number of species [71], colourful female ornaments are positively associated with residual mass and immune response. A number of empirical studies have suggested these associations might result from more colourful individuals having better foraging abilities or capacity to use micronutrients (carotenoids) ([46,72,73], but see [74]), increased access to nutritional resources through aggressive displays (melanin) ([75], but see [42]), or greater availability of energy storages (structural) ([47], but see [46]). Links between female coloration with residual mass and immune response may have emerged through selective pressures acting on females themselves (as a result of specific selective pressures), males (with correlative consequences on females) or both [76]. Although we cannot directly conclude that female coloration has a signalling role, our results suggest
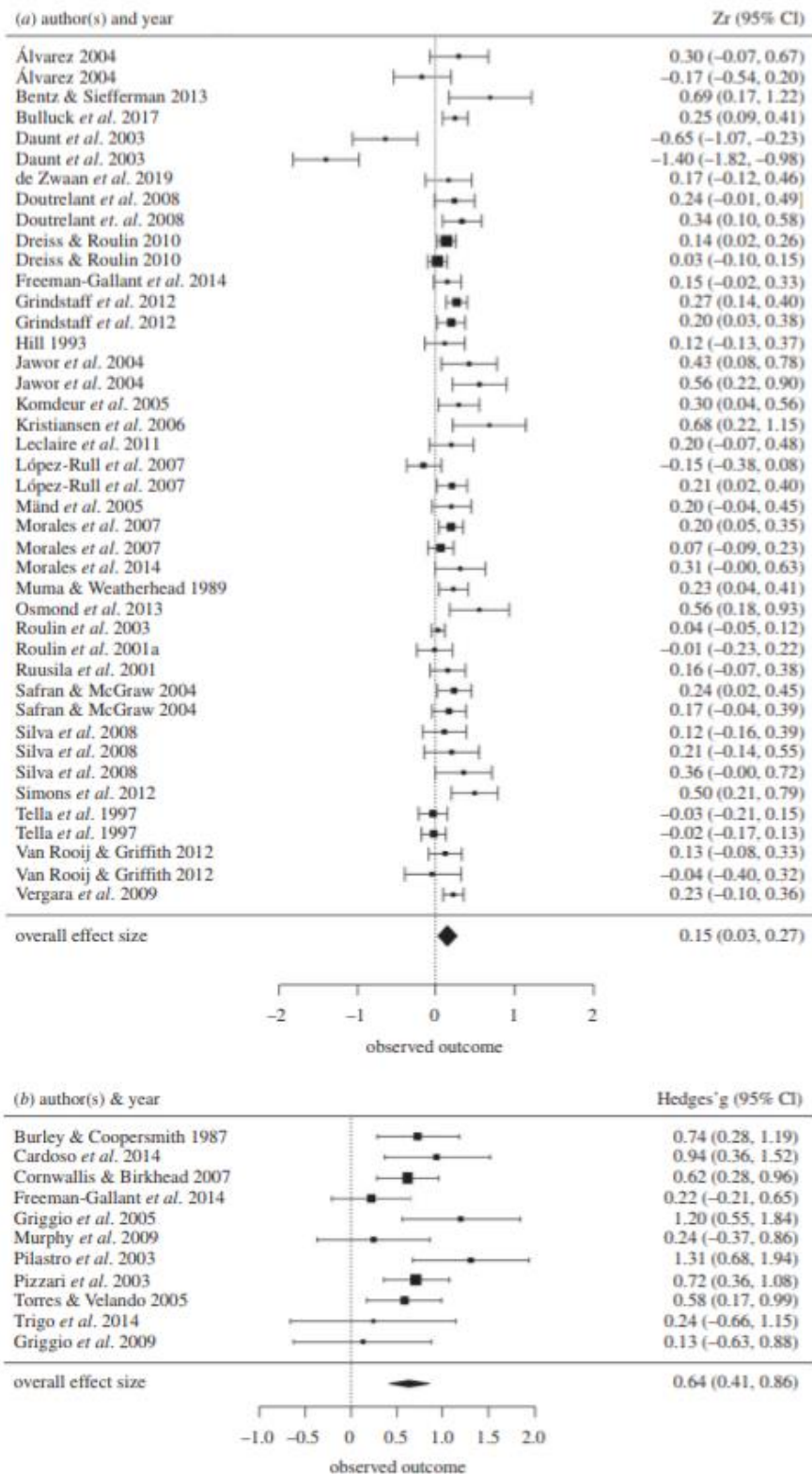
(a) author(s) and year	Zr (95% CI)
Álvarez 2004	0.30 (−0.07, 0.67)
Álvarez 2004	−0.17 (−0.54, 0.20)
Bentz & Siefferman 2013	0.69 (0.17, 1.22)
Bulluck et al. 2017	0.25 (0.09, 0.41)
Daunt et al. 2003	−0.65 (−1.07, −0.23)
Daunt et al. 2003	−1.40 (−1.82, −0.98)
de Zwaan et al. 2019	0.17 (−0.12, 0.46)
Doutrelant et al. 2008	0.24 (−0.01, 0.49]
Doutrelant et. al. 2008	0.34 (0.10, 0.58)
Dreiss & Roulin 2010	0.14 (0.02, 0.26)
Dreiss & Roulin 2010	0.03 (−0.10, 0.15)
Freeman-Gallant et al. 2014	0.15 (−0.02, 0.33)
Grindstaff et al. 2012	0.27 (0.14, 0.40)
Grindstaff et al. 2012	0.20 (0.03, 0.38)
Hill 1993	0.12 (−0.13, 0.37)
Jawor et al. 2004	0.43 (0.08, 0.78)
Jawor et al. 2004	0.56 (0.22, 0.90)
Komdeur et al. 2005	0.30 (0.04, 0.56)
Kristiansen et al. 2006	0.68 (0.22, 1.15)
Leclaire et al. 2011	0.20 (−0.07, 0.48)
López-Rull et al. 2007	−0.15 (−0.38, 0.08)
López-Rull et al. 2007	0.21 (0.02, 0.40)
Mänd et al. 2005	0.20 (−0.04, 0.45)
Morales et al. 2007	0.20 (0.05, 0.35)
Morales et al. 2007	0.07 (−0.09, 0.23)
Morales et al. 2014	0.31 (−0.00, 0.63)
Muma & Weatherhead 1989	0.23 (0.04, 0.41)
Osmond et al. 2013	0.56 (0.18, 0.93)
Roulin et al. 2003	0.04 (−0.05, 0.12)
Roulin et al. 2001a	−0.01 (−0.23, 0.22)
Ruusila et al. 2001	0.16 (−0.07, 0.38)
Safran & McGraw 2004	0.24 (0.02, 0.45)
Safran & McGraw 2004	0.17 (−0.04, 0.39)
Silva et al. 2008	0.12 (−0.16, 0.39)
Silva et al. 2008	0.21 (−0.14, 0.55)
Silva et al. 2008	0.36 (−0.00, 0.72)
Simons et al. 2012	0.50 (0.21, 0.79)
Tella et al. 1997	−0.03 (−0.21, 0.15)
Tella et al. 1997	−0.02 (−0.17, 0.13)
Van Rooij & Griffith 2012	0.13 (−0.08, 0.33)
Van Rooij & Griffith 2012	−0.04 (−0.40, 0.32)
Vergara et al. 2009	0.23 (−0.10, 0.36)
overall effect size	0.15 (0.03, 0.27)

(b) author(s) & year	Hedges'g (95% CI)
Burley & Coopersmith 1987	0.74 (0.28, 1.19)
Cardoso et al. 2014	0.94 (0.36, 1.52)
Cornwallis & Birkhead 2007	0.62 (0.28, 0.96)
Freeman-Gallant et al. 2014	0.22 (−0.21, 0.65)
Griggio et al. 2005	1.20 (0.55, 1.84)
Murphy et al. 2009	0.24 (−0.37, 0.86)
Pilastro et al. 2003	1.31 (0.68, 1.94)
Pizzari et al. 2003	0.72 (0.36, 1.08)
Torres & Velando 2005	0.58 (0.17, 0.99)
Trigo et al. 2014	0.24 (−0.66, 1.15)
Griggio et al. 2009	0.13 (−0.63, 0.88)
overall effect size	0.64 (0.41, 0.86)

Figure 2. Association of colourful female traits with reproductive performance (a) and male-mate preference (b) (effect sizes ± 95% confidence intervals).

that there is the opportunity for female colour to evolve as a condition-dependent signal, which may be particularly relevant for males when it conveys information on fecundity.

Colourful females had better reproductive performance, measured as clutch size. Interestingly, only carotenoid-dependent colorations and integuments (but not feathers) were associated with clutch size. Hence, carotenoid-dependent colours in integumentary body parts, which can be dynamically updated in response to changes in condition [77], may be signals of direct benefits for males in the form of female fecundity [24]. This result is in line with the fact that colour and condition were positively associated and together suggest that females expressing carotenoid-dependent colourful traits can acquire and allocate resources to both ornamentation and reproduction. Like carotenoid-dependent coloration, clutch size is a phenotypically plastic trait that is sensitive to fluctuations in nutritional resource availability [78]. Thus, both can be linked to environmental sources of variation. However, the fact that carotenoid-dependent colours are repeatable despite being dynamic suggests that genetic variation in, for example, the capacity to acquire, absorb, transport, metabolize and store (micro)nutrients and/or withstand resource fluctuation may also play a role linking this type of colour with reproductive performance and condition [79–82]. Interestingly, a previous review found evidence for a positive association between female colour and reproductive performance in birds, but that result was not replicated in fish and became inconclusive (in birds) when only carotenoid-dependent colours were considered [83]. In addition, that review did not include a quantitative synthesis, so it is unclear whether information on the influence of moderator variables would have affected the interpretation of those results. Contrasting results between fish and birds may be because most of the avian species studied so far exhibit a monogamous mating system. The potential role played by the mating system on these results warrants further examination. Hence, in monogamous birds, male choosiness might be favoured when parental duties are shared by both sexes, reducing opportunities for the male to re-mate [23].

In studies of sexual selection, male-mate choice is still little explored. Accumulated evidence analysed here, mainly from monogamous species, supports the idea that males prefer females that display more colourful carotenoid-dependent traits. The fact that we found carotenoid-dependent colours in females to be related to both condition and reproductive performance suggests that those ornaments may indeed inform about direct benefits for the reproductive partner. However, the association between colourful traits and residual body mass found in females—which may conceivably be directly assessed by the males—highlights the opportunity for further studies to separate the potential confounding effects of these two characteristics on males' preference. In general, more experimental studies evaluating male preferences for all three main types of female coloration are required to better understand the generality of this result.

This meta-analytic study provides support for the idea that colourful attributes in female birds are linked to condition and reproductive performance and may be maintained by sexual selection through male-mate choice. Accumulated evidence from birds, mainly from species with socially monogamous mating systems and biparental care, suggests that when direct benefits for pairing with a high-quality mate can be accrued, males may choose sexual partners based on information provided by coloured traits.

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