UNIVERSIDAD MICHOACANA
DE SAN NICOLÁS DE HIDALGO

Instituto de Investigaciones sobre los Recursos Naturales

Programa Institucional de Doctorado en Ciencias Biológicas

"Efecto de la inmunocompetencia materna y del ambiente de incubación sobre la respuesta inmunitaria innata de crías de *Lepidochelys olivacea*"

TESIS

Para Obtener el Grado de

Doctora en Ciencias Biológicas

Opción: Recursos Bióticos

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Morelia, Michoacán. Mayo de 2026

DEDICATORIA

Dedico esta tesis a mi madre por todos los esfuerzos que ha hecho a lo largo de mi desarrollo profesional y personal. Gracias a su apoyo y compañía he podido alcanzar varias metas. Tus consejos me han alentado para llegar hasta este punto.

AGRADECIMIENTOS

Con el apoyo de la Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) becario No. 817553 y de la Coordinación de la Investigación Científica de la UMSNH.

Este proyecto de investigación se pudo realizar gracias a la asesoría de la Dra. Esperanza Meléndez Herrera y a los consejos y apoyo de mi comité tutoral conformado por el Dr. Omar Chassin Noria, a la Dra. Rosa Elvira Núñez Anita quien además me apoyo con los experimentos de capacidad hemolítica en su laboratorio, a los doctores Bryan Víctor Phillips Farfán y Ernesto Vicente Vega Peña por su ayuda en los análisis estadísticos.

Extiendo mi gratitud a la Dra. Ma. Antonia Herrera Vargas por su ayuda durante las salidas de campo y en los experimentos en laboratorio.

A Saúl González de la Luz por sus consejos y ayuda durante parte del trabajo en campo.

A las estudiantes Dayana Marian Cambrón Reyes, Ana Karen Pitacua Guzmán y America Dalaith Pineda Díaz, por su ayuda en el procesamiento de las muestras y elaboración de experimentos de expresión génica.

Agradezco a la M. en C. Martha Harfush Meléndez por su dedicación y apoyo durante el trabajo de campo en el Santuario Playa la Escobilla, Oaxaca y al M.V.Z. Rafael Villanueva por facilitarnos información para la planificación de los muestreos y por su apoyo durante nuestra estancia en el Santuario.

Gracias a todos los integrantes del “Centro Ecoturístico La Escobilla” por su cariño y apoyo durante el tiempo que estuvimos realizando los muestreos. Particularmente agradezco la participación en el desarrollo de la logística en campo a don Eradio, don Sóstenes, Manuel, Eloísa, Rosita, Erica, Tere, Flora y Edgar. Mi admiración, respeto y cariño están con todos los integrantes del Centro Ecoturístico.

CONTENIDO

I. RESUMEN GENERAL

Las tortugas marinas enfrentan múltiples amenazas, entre ellas el saqueo de nidos, la depredación y el aumento de la temperatura ambiental. Adicionalmente, el calentamiento global está favoreciendo un aumento en la exposición a patógenos en los sitios de anidación. Para protegerse contra las infecciones, el desempeño inmunitario de las crías es crucial. Esto implica erradicar eficientemente al desafío infeccioso, evitando el daño de los tejidos propios (i.e., inmunocompetencia). En las crías, el fenotipo inmunitario está influenciado tanto por el ambiente de incubación como por rasgos maternos. Sin embargo, aún no está claro hasta qué punto estos factores moldean las respuestas inmunitarias inducibles, por ejemplo, ante una infección bacteriana. Con la finalidad de identificar cómo estos factores promueven el desarrollo de fenotipos inmunitarios óptimos en las crías, en el primer capítulo se exploró el efecto de la talla y la capacidad hemolítica materna sobre la capacidad hemolítica en crías de tortuga marina *Lepidochelys olivacea* expuestas a un desafío con lipopolisacárido (LPS) bacteriano. Los resultados muestran una relación positiva de la masa y la capacidad hemolítica materna con la capacidad hemolítica de las crías, mientras que el reto con LPS no indujo cambios en la respuesta de las crías. Esto sugiere que la capacidad hemolítica de las crías está determinada principalmente por la influencia materna, independientemente del ambiente de incubación y de la exposición a un reto, lo que les confiere un mecanismo de defensa estable durante etapas tempranas de la vida.

El segundo capítulo evaluó el efecto del ambiente y su interacción con el efecto materno sobre la expresión de marcadores de respuesta inmunitaria y defensa oxidante en el hígado y bazo de crías en respuesta a LPS. Para ello, se empleó un diseño de nidada dividida en la que, la mitad de los huevos de cada hembra fueron incubados en viveros sin sombra y la otra mitad en viveros sombreados. El tamaño corporal y la capacidad hemolítica materna se evaluaron como predictores de la expresión génica de las crías. Los resultados muestran que, en el hígado, el LPS incrementó la expresión de *interleucina-1β* en ambas condiciones de incubación mientras que únicamente incrementó la expresión de *interleucina-6* en las crías incubadas en viveros sin sombra. La expresión de las enzimas antioxidantes y del marcador pro-apoptótico *caspasa-3* fue

mayor en las crías incubadas en viveros sin sombra. En el bazo, la expresión de *catalasa* fue mayor en las crías incubadas en viveros sombreados, en contraste con la expresión de *caspasa-3*, que fue mayor en las crías incubadas en viveros sin sombra. Los efectos maternos se identificaron únicamente en el bazo, ya sea en interacción con el ambiente de incubación o de manera independiente, modulando la expresión de *catalasa* y *heat shock protein-90*. Los resultados sugieren que la respuesta inmunitaria en crías está determinada por la acción complementaria de los efectos maternos y el ambiente de incubación, integrando una defensa innata estable con capacidad de ajuste frente a retos inmunitarios. Esta interacción podría favorecer ajustes fenotípicos tempranos que incrementen la inmunocompetencia de las crías en ambientes variables y bajo escenarios de cambio climático.

Palabras clave: cambio climático, capacidad hemolítica, lipopolisacárido, respuesta inflamatoria, respuesta antioxidante

II. RESUMEN GENERAL (ABSTRACT)

Sea turtles face multiple threats, including nest poaching, predation, and increasing environmental temperatures. Additionally, global warming is promoting increased exposure to pathogens at nesting sites. To protect against infections, hatchlings' immune performance is crucial. This involves efficiently eliminating infectious challenges while avoiding damage to self-tissues (i.e., immunocompetence). In hatchlings, the immune phenotype is influenced by both the incubation environment and maternal traits. However, it is still unclear to what extent these factors shape inducible immune responses, for example, in response to a simulated bacterial infection. In order to identify how these factors promote the development of optimal immune phenotypes in hatchlings, the first chapter explored the effect of maternal size and hemolytic capacity on hatchlings hemolytic capacity in olive ridley sea turtle *Lepidochelys olivacea* exposed to a bacterial lipopolysaccharide (LPS) challenge. The results show a positive relationship between maternal mass and hemolytic capacity with hatchling's hemolytic capacity, whereas the LPS challenge did not induce changes in hatchling responses. This suggests that hatchlings' hemolytic capacity is mainly determined by maternal influence, independently of the incubation environment and exposure to a challenge, conferring a stable defense mechanism during early life stages.

The second chapter evaluated the effect of the incubation environment and its interaction with maternal effects on the expression of immune response and oxidative defense markers in the liver and spleen of hatchlings in response to LPS. Briefly, a split-clutch design was used in which, half clutch from each female was incubated in an unshaded hatchery while the other was incubated in a shaded hatchery. Maternal body size and hemolytic capacity were evaluated as predictors of gene expression in hatchlings. The results show that, in the liver, the LPS challenge induced higher expression of *interleukin-1β* in both incubation conditions while only enhanced *interleukin-6* expression in hatchlings incubated in unshaded hatcheries. Expression of the antioxidant enzymes and the pro-apoptotic marker *caspase-3* was higher in hatchlings incubated in unshaded hatcheries. In the spleen, *catalase* expression was higher in shaded incubated hatchlings,

in contrast with *caspase-3* expression which was higher in unshaded incubated hatchlings. Maternal effects were identified only in the spleen, either in interaction with the incubation environment or independently, modulating the expression of *catalase* and *heat shock protein-90*. The results suggest that hatchlings immune response is determined by the complementary action of maternal effects and the incubation environment, integrating a stable innate defense with the ability to adjust to immune challenges. This interaction may favor early phenotypic adjustments that enhance hatchlings immunocompetence in changing environments and under climate change scenarios.

III. INTRODUCCIÓN GENERAL

Las tortugas marinas son especies amenazadas por diversos factores, por ejemplo, el saqueo de nidos, las altas temperaturas derivadas del cambio climático y el aumento de las enfermedades infecciosas (Alfaro et al., 2008; Mashkour et al., 2020). Estas últimas son de preocupación prioritaria debido a que su incidencia aumenta en paralelo con las temperaturas, exacerbando el declive de poblaciones ya susceptibles (Alfaro et al., 2008). Para las tortugas marinas, las bacterias son los microrganismos más comunes y pueden infectar tanto a los individuos de silvestres como en cautiverio (Glazebrook y Campbell, 1990; Raidal et al., 1998). En tortuga golfina, la exposición a bacterias puede ocurrir desde muy temprano en la vida. En playas de arribada, la alta mortandad de huevos que generan las hembras al excavar nidadas de arribadas previas favorece la proliferación bacteriana (Bernardo y Plotkin 2007; Bezy et al., 2015; Cornelius et al., 1991; Madden et al., 2008).

Para protegerse de microorganismos infecciosos, la respuesta inmunitaria innata es crucial en los reptiles. Actúa como la primera línea de defensa y es la más robusta (Nash y Ryan, 2023; Zimmerman et al., 2010). Está conformada por una red compleja que involucra órganos como el bazo y el hígado, células fagocíticas y moléculas líticas y citocinas proinflamatorias que se activan cuando algunos receptores en las membranas celulares reconocen patrones moleculares asociados a patógenos (PAMPs) o a daño celular (DAMPs) con el fin de controlar la infección (Nash y Ryan, 2023; Takuechi y Akira, 2010; Zimmerman et al., 2010).

Durante este proceso, se produce un aumento de moléculas altamente reactivas conocidas como radicales libres. Si bien estas moléculas contribuyen en la destrucción de los agentes infecciosos, pueden provocar daño en estructuras como el ADN, proteínas y lípidos cuando se producen en exceso o durante periodos prolongados (Freeman y Crapo, 1982; Miller y Zachary, 2017).

Una respuesta inmunitaria eficiente requiere neutralizar la infección y al mismo tiempo proteger los tejidos propios contra el daño oxidante. La respuesta antioxidante de enzimas como la catalasa, superóxido dismutasa y glutatión reductasa involucran

reacciones de óxido-reducción que mantienen el equilibrio redox y amortiguan el daño tisular (Freeman y Crapo, 1982; Zhang et al., 2019). A esta respuesta integral se le denomina inmunocompetencia (Chaplin, 2010; Holsapple y Kaminski, 2005).

La competencia inmunitaria en los reptiles puede verse afectada por la temperatura, una variable ambiental de gran relevancia. En tortugas de agua dulce se ha observado que las crías que experimentan temperaturas de incubación elevadas presentan un menor desempeño inmunitario frente a infecciones bacterianas (Dang et al., 2015). En tortugas marinas, la reubicación de nidos a viveros protegidos con malla sombra se considera una práctica útil que promueve el desarrollo de machos y favorece el porcentaje de supervivencia, la aptitud física y talla corporal de las crías, posiblemente asociado que las temperaturas en estos viveros se encuentran cercanas a las óptimas para la especie (Robledo-Avila et al., 2022). Además, se ha observado que los viveros protegidos con malla sombra promueven un mayor desarrollo esplénico en las crías (Robledo-Avila et al., 2022). Aunque las crías utilizadas en este estudio no fueron retadas inmunitariamente, en los reptiles el bazo ha sido descrito como el órgano más importante para la defensa contra bacterias extracelulares (Zhang et al., 2016). Por otra parte, se observó que las crías provenientes de nidos sin sombra llegan a experimentar temperaturas de incubación superiores a 35°C (Robledo-Avila et al., 2023; García-Bucio et al., 2025). Las crías de *L. olivacea* incubadas en nidos sin sombra presentaron una mayor actividad prooxidante y capacidad antioxidante, sugiriendo que su riesgo de sufrir estrés oxidante es mayor en comparación con crías incubadas en viveros sombreados (Robledo-Avila et al., 2023). Algo similar ocurrió en tortugas de caparazón blando (*Pelodiscus sinensis*), donde la expresión de enzimas antioxidantes aumentó en respuesta a temperaturas elevadas, mientras que la actividad enzimática involucrada con la defensa antioxidante disminuyó debido al estrés térmico (Zhang et al., 2019).

Por último, un estudio realizado en crías de *L. olivacea* evaluó la competencia inmunitaria ante el mitógeno fitohemaglutinina, reportó que la respuesta inflamatoria de crías incubadas en viveros sombrados fue menor y más lenta que la de aquéllas incubadas en nidos sin sombra (García-Bucio et al., 2025).

Si bien queda claro que la inmunocompetencia en tortugas esta influenciada por la temperatura, aún existe un rezago significativo en el conocimiento de los factores que moldean el desarrollo de la respuesta inmunitaria en las tortugas marinas.

En diversas especies de reptiles, se ha observado que la competencia inmunitaria de las crías también puede ser moldeada por rasgos maternos. Aspectos como la talla corporal, la reproducción y la respuesta inmunitaria, son procesos esenciales para los individuos, sin embargo, demandan altos costos energéticos. En este contexto, una hembra reproductiva con recursos reducidos, para evitar comprometer su sistema inmunitario y arriesgar su supervivencia, debe hacer negociaciones fisiológicas, limitando el aporte de moléculas inmunitarias y energéticas hacia su progenie (Houston et al., 2007; Uller et al., 2006). La incorporación de estas moléculas al embrión puede ocurrir durante la vitelogénesis (Virgin et al., 2022) con el fin de beneficiar a las crías y permitirles invertir su energía en elementos cruciales del crecimiento (Karell et al., 2008).

Estudios previos realizados en tortugas marinas de la especie *Caretta caretta*, reportan que hembras con mayor talla corporal y expuestas a infección por parásitos, favorecen el desarrollo de crías más grandes (Lockley et al., 2020). En tortugas de desierto *Gopherus agassizii*, las crías de progenitores infectados presentan mayores concentraciones de anticuerpos como la inmunoglobulina (Ig)G e IgM (Schumacher et al., 1999). En *L. olivacea* se ha observado una asociación positiva entre la cantidad de células inmunitarias maternas y la longitud corporal de sus crías provenientes de nidos sin sombra (García-Bucio et al., 2025). Adicionalmente, indicadores bioquímicos y de talla materna como el colesterol, la globulina y el ancho recto del caparazón se asociaron positivamente con la programación inmunitaria de crías incubadas en nidos sombreados, favoreciendo la expresión esplénica de marcadores inmunitarios como *tlr4*, *pi3k* e *il-6* y la expresión hepática de *tlr4* (García-Bucio et al., en revisión). La relación entre la talla materna y la respuesta inmunitaria de las crías también ha sido observada en la serpiente de quilla (*Tropidonophis mairii*), donde la talla corporal de las hembras se asocia positivamente con la configuración inmunitaria celular de sus crías (Brown y Shine 2016).

Para implementar mejoras en las prácticas de conservación, es indispensable realizar estudios eco-inmunológicos que nos permitan identificar los factores que moldean la respuesta inmunitaria innata de neonatos de tortuga marina, un aspecto crucial para la supervivencia de los individuos. A pesar de la relevancia ecológica que las bacterias tienen como agentes infecciosos para las tortugas marinas, su efecto sobre la respuesta inmunitaria en neonatos aún no se ha explorado y tampoco se ha evaluado si la preparación inmunitaria materna prepara la respuesta inmunitaria de sus crías. Hasta ahora únicamente se ha identificado un efecto de factores nutricionales y de talla corporal materna sobre la talla y la configuración inmunitaria de sus crías, no obstante, la contribución materna ante un reto inmunitario en las crías aún no ha sido descrito. En este contexto, el presente trabajo propone un abordaje novedoso orientado a identificar las variables que moldean la inmunocompetencia de las crías de tortuga marina *Lepidochelys olivacea* a la emergencia del nido. La primera parte del proyecto aborda la relación entre los factores maternos y la respuesta inmunitaria de las crías ante un reto inmunitario ecológicamente relevante como el lipopolisacárido (LPS) bacteriano. Particularmente, explora cuáles factores morfológicos y de inmunocompetencia materna podrían determinar la respuesta inmunitaria de las crías. La segunda parte del proyecto se orienta en entender el efecto del ambiente de incubación sombreado y sin sombra y de su interacción con factores maternos, sobre la inmunocompetencia de crías desafiadas con LPS.

IV. PLANTEAMIENTO DE HIPÓTESIS

Las crías de *L. olivacea* incubadas en viveros sombreados y provenientes de madres con mayor talla corporal y mayor respuesta inmunitaria presentan una mayor respuesta inmunitaria innata frente al reto con LPS y exhiben una mayor expresión de enzimas antioxidantes.

V. OBJETIVOS

V.I. General

Evaluar el efecto materno y del ambiente de incubación sobre la respuesta inmunitaria y la defensa antioxidante de crías incubadas en viveros sin sombra (SS) y sombreados (S) sometidas a un reto inmunitario simulado con lipopolisacárido (LPS) de *Escherichia coli*.

V.II. Particulares

V.II.I. Determinar el efecto de la talla corporal y la respuesta inmunitaria materna sobre la respuesta inmunitaria innata de crías de *Lepidochelys olivacea*.

V.II. II. Determinar el efecto del ambiente de incubación y de su interacción con la talla y la respuesta inmunitaria materna sobre la respuesta inmunitaria innata y la defensa antioxidante de crías de *Lepidochelys olivacea* a la emergencia.

CAPÍTULO 1. MATERNAL TRAITS ARE ASSOCIATED WITH HEMOLYTIC CAPACITY IN SEA TURTLE HATCHLINGS, INDEPENDENTLY OF SYSTEMIC IMMUNE STIMULI

1) Resumen (Abstract)

Las enfermedades infecciosas constituyen una gran amenaza para la supervivencia de las tortugas marinas. En las crías, la respuesta inmunitaria se configura durante el desarrollo temprano y está fuertemente influenciada por el ambiente de incubación y aunque en algunos reptiles puede estar influenciada por rasgos maternos, en tortugas marinas aún se ha esclarecido. La capacidad hemolítica es un componente clave del sistema inmunitario, estable al ambiente de incubación, lo que sugiere que este rasgo podría ser moldeado por características maternas. En este estudio se evaluó la relación entre la capacidad hemolítica de crías retadas con lipopolisacárido (LPS) bacteriano y características maternas que incluyeron la capacidad hemolítica y la talla corporal. Durante dos años se colectaron muestras sanguíneas y parámetros morfológicos de hembras anidantes. Las nidadas fueron reubicadas en viveros sombreados y a la emergencia, una cría de cada nido fue inoculada intraperitonealmente con solución de fosfatos (PBS) y la otra con LPS. Después de 4 horas, las crías fueron sometidas a eutanasia y se obtuvieron muestras sanguíneas. Los resultados muestran que las madres con mayor masa y capacidad hemolítica promueven el desarrollo de crías con mayor capacidad hemolítica, no obstante, el largo recto de caparazón materno estuvo inversamente relacionado con la capacidad lítica de las crías. El reto con LPS no modificó significativamente la capacidad hemolítica de las crías. Debido al estado de conservación de la especie y a dificultades técnicas asociadas a estudios ecoinmunológicos, el tamaño de muestra fue reducido, no obstante, los hallazgos sugieren una influencia materna sobre la función inmunitaria de las crías. También que la capacidad hemolítica de las crías es un rasgo inmunitario robusto que las protege durante etapas tempranas de la vida.

Este artículo está publicado en la revista Journal of Experimental Zoology Part A: Ecological and Integrative Physiology con doi:10.1002/jez.70032, cumple con el primer objetivo particular del proyecto de investigación y es uno de los requisitos de titulación para obtener el grado de Dra. en Ciencias bajo el Programa Institucional de Doctorado en Ciencias Biológicas, UMSNH.

CAPÍTULO 2. THE INCUBATION ENVIRONMENT AND MATERNAL TRAITS PROGRAM IMMUNE AND CELLULAR STRESS RESPONSES IN SEA TURTLE HATCHLINGS

1) Abstract

Global warming is altering incubation temperatures and pathogen exposure of sea turtles. Because hatchling immune phenotype is a developmentally plastic trait shaped by both incubation conditions and maternal inputs, understanding how these factors interact to regulate inducible immune responses is critical. Here, hepatic and splenic expression of proinflammatory cytokines, antioxidant enzymes, thermal-stress molecules and a proapoptotic marker were quantified in *Lepidochelys olivacea* hatchlings exposed to a lipopolysaccharide (LPS) challenge. Using a split-clutch design, eggs from the same females were incubated in unshaded and shaded hatcheries. Maternal body size and hemolytic capacity were assessed as predictors of hatchlings' gene expression. LPS challenge increased hepatic *il-1β* expression in hatchlings from both incubation environments. In contrast, expression of antioxidant enzymes was reduced in hatchlings from shaded hatcheries in the liver, accompanied by lower splenic *casp-3* expression. Multiple-comparison analyses indicated that hepatic *il-6* and *gr* expression was reduced in PBS-treated hatchlings from shaded nests, whereas *casp-3* expression was increased in LPS unshaded conditions. Neither incubation environment nor LPS challenge significantly affected *tnf-α*, *hsp70* or *hsp90* expression in either organ. Maternal effects were gene-specific and restricted to the spleen. Offspring from wider females exhibited higher expression of *hsp90*, independently from the incubation environment. In contrast, maternal body mass and hemolytic capacity exerted incubation environment-dependent effects on hatchling *hsp90* expression. Additionally, maternal mass was positively associated in interaction with the incubation environment with hatchlings splenic *cat* expression. These findings indicate that the incubation environment does not necessarily alter the ability of hatchlings to mount an inducible inflammatory response to LPS, but instead programs the early metabolic and cellular stress configurations and governs the expression of maternal effects.

2) Introduction

Global warming poses significant challenges for sea turtle development, including rising incubation temperatures, flooding, intense rainfalls and drought (Hawkes et al., 2007; Maurer et al., 2021; Rivas et al., 2018). Early-life immune function is a highly plastic trait shaped by both the developmental environment and maternal influences in oviparous ectotherms (Brown and Shine, 2016; Dang et al., 2015; García-Bucio et al., 2025; Lu et al., 2021). In sea turtles, the immune configuration is established during embryogenesis and early post-hatchling stages (Brown and Shine, 2018; Freedberg et al., 2008; Leivesley and Rollingson, 2021), and may determine short-term survival during the critical dispersal period. Understanding the mechanisms that generate variation in inducible immune phenotypes is therefore essential for predicting population responses to environmental change.

Incubation conditions are known to influence physiological, behavioral and immune traits in sea turtles (Robledo-Avila et al., 2022; Robledo-Avila et al., 2023). Experimental work in *Lepidochelys olivacea* has shown that the incubation environment alters basal immune gene expression in the liver and spleen, suggesting early-life programming of immune function. Specifically, hatchlings incubated in shaded hatcheries exhibited elevated splenic and hepatic expression of previously described immune mediators such as toll like receptor 4 (*tlr4*), phosphatidylinositol 3-kinase (*pi3k*), ras-related C3 botulinum toxin substrate 1 (*rac1*) and nuclear factor kappa-b (*nfkb*), as well increased expression of heat shock protein 70 (*hsp70*) and *hsp90* compared with hatchlings from the unshaded condition (Robledo-Avila et al., 2023; García-Bucio et al., in review). Because shading simultaneously modifies temperature and moisture, and strongly biases sex ratios (unshaded = predominantly female; shaded = predominantly male), follow-up work assessed whether temperature-dependent sex explained these transcriptional differences (García-Bucio et al., in review). Under controlled feminizing and masculinizing temperature regimes, only *pi3k* and *rac1* expression increased at feminizing temperatures. This suggests that immune gene expression differences between hatchery conditions cannot

be attributed solely to temperature-linked sex and likely reflect additional incubation-related factors (García-Bucio et al., in review).

Maternal traits also contribute to hatchling immune phenotype. Recent evidence shows that maternal life-history characteristics, such as hemolytic capacity and body size, predict offspring's humoral response to foreign erythrocytes (Chávez-Salazar et al., 2025). Moreover, maternal effects on both basal immune configuration and hatchling body size appear to be context-dependent, varying with the incubation environment (García-Bucio et al., 2025; García-Bucio et al., in review).

Here, using the same split-clutch experimental design, splenic and hepatic expression of proinflammatory cytokines tumor necrosis factor-alpha (*tnf-α*), interleukin 1β (*il-1β*) and *il-6*, antiantioxidant enzymes superoxide dismutase (*sod*), catalase (*cat*) and glutation reductase (*gr*), heat shock proteins (*hsp70* and *hsp90*) and the proapoptotic enzyme caspase 3 (*casp-3*) was quantified in *L. olivacea* hatchlings subjected to a lipopolysaccharide (LPS) challenge. The aim was to evaluate how incubation and maternal traits jointly shape inducible immune phenotypes and to test whether maternal effects on immune function are expressed or masked under immune activation. Based on the previous results, the hypothesis was that the turtles from shaded hatcheries would show an enhanced proinflammatory profile, increased thermal-stress related protection and diminished proapoptotic expression in response to LPS challenge compared to unshaded-incubated offspring.

3) Material and methods

Animal handling and sampling procedures were approved by Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) through the General Department of Wildlife under license number SPARN/DGVS/01278/24 in compliance with Mexican regulations (NOM-059-SEMARNAT-2010, 2012). All efforts to minimize possible pain, injury, anxiety or stress were performed.

3.1 Study area and sampling of females

Between the 2nd and 4th night of 7th arribada from season 2023-2024, 10 nesting females were selected within a 500 m range from the middle beach zone at Playa La Escobilla Sanctuary, Oaxaca, México (15° 43,4′ N, 96° 44.3′ W). During egg laying, morphological measurements from each nesting female were obtained. The straight carapace length (SCL) and width (SCW) were measured using a caliper (Titanium, max = 100 cm, d = 0.02 mm) and the curved carapace length (CCL) and width (CCW) using a 100 cm measuring tape. Once the nesting female finished ovipositing, the body mass was weighted with a Roman tubular balance (Trupper, max = 50 kg, d = 0.5 kg). After proper asepsis, a 5 ml blood sample was collected from the sinus vein using a 10 cc syringe (21 G × 32 mm; BD Plastipak, Becton Dickinson) to obtain plasma. Its hemolytic capacity was evaluated in the laboratory as an indicator of maternal immune function as previously described (Chávez-Salazar et al., 2025). Briefly, maternal hemolytic capacity was determined by incubating plasma 1:2 in Dextrose-Gelatin-Veronal buffer (Lonza, Walkersville, MD, USA) with a 2% suspension of sheep erythrocytes (SRBC 2%) for 60 minutes at room temperature. A positive control with 100% lysis was also included using SRBC 1% with Triton X-100 and a negative control by incubating 100 µl of SRBC 2% with 100 µl of Dextrose-Gelatin-Veronal buffer. Following incubation, samples were centrifuged at 2500 rpm for 3 minutes and in a clear 96-well plate, samples were run in duplicate by transferring 150 µl of the supernatant. Absorbance was measured at 540 nm using a Varioskan Multimode microplate reader (Thermo).

3.2 Clutch split and hatchling sampling

The clutches of each nesting female were divided equally and placed in individual plastic bags immediately after oviposition. To minimize developmental disturbances, egg rotation was strictly avoided, and all half-clutches were reburied within 2 h in nests 50 cm deep and spaced 1 m apart, following the recommended guidelines for *L. olivacea* (Sarti et al., 2006). Clutch relocation and hatcheries construction were conducted by the staff of “Centro Ecoturístico La Escobilla”. A half of each clutch (n=10) was placed in an open-air fenced

hatchery, while the remaining half (n=10) was reburied in a 405 m² shaded hatchery covered with 50% shade mesh.

A few days before the end of the incubation period in both unshaded and shaded hatcheries, individual fences were placed around each nest and monitored frequently for hatchling emergence. Once the first two hatchlings from each nest emerged, one individual received an intraperitoneal injection of phosphate-buffered saline (PBS, 0.1 M), while the other was injected intraperitoneally with a dose of LPS from *Escherichia coli* (0.0025 mg endotoxin per gram of body mass; Sigma, St. Louis, MO, USA) dissolved in PBS, to simulate infection and elicit an innate immune response (Fig. 1; Chávez-Salazar et al., 2025). Afterwards, the hatchlings were maintained under controlled conditions and euthanized within 4h in accordance with Mexican regulations (NOM-033-SAG/ZOO-2014). Briefly, the spleen and liver were collected and preserved in RNAlater solution (Sigma). All samples were stored at -70°C and in the laboratory, RNA from each organ was obtained in TRIzol™ reagent (Invitrogen) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the M-MLV Reverse Transcriptase kit (Promega). Messenger RNA (mRNA) expression of the proinflammatory cytokines: *tnf-α*, *il-1β* and *il-6*; the antioxidant enzymes: *sod*, *cat* and *gr*; heat shock proteins *hsp70* and *hsp90*, as well as the proapoptotic enzyme *casp-3* was quantified using a termocycler (BioRad, Model: CFX96™) and the iTaq Universal SYBR® Green Supermix kit. Relative mRNA expression was quantified with the delta cycle threshold (ΔCT) method, using the small subunit of gene 18S rRNA (*r18s*) as the control. All primers used are reported in table 1. The samples were run in duplicate and all primers were validated before use. Transcripts with CT values over 35 were considered as undetectable (ND) and excluded from analysis. Their number per gene is reported in the results section. In samples where the replicate was ND, while the other amplified near the detection limit (CT < 35), the detectable replicate was selected and the mean CT value was calculated. To determine sex, the left gonad-mesonephros complex of all hatchlings were obtained and stored in 10% formalin. By histological techniques, the samples were processed based on previous studies criteria (Herrera-Vargas et al., 2017).

3.3 Statistical analyses

The primary objective of this study was to assess the effects of the incubation environment and its interaction with maternal traits on hatchling immune function. Briefly, data normality was assessed using the Shapiro-Wilk test ($p \leq 0.05$). Additionally, using Pearson correlation tests, maternal parameters were evaluated to identify which were covariates. Using the delta cycle threshold (ΔCt) of hepatic and splenic immune markers, mixed linear models were performed. First, models were constructed individually for each hepatic and splenic marker to evaluate the influence of the incubation environment and immune challenge. The incubation environment and immune challenge were included as fixed effects and nest identity as a random effect. Because maternal body size and immune characteristics may contribute to hatchling immune responses, additional models were developed to examine these relationships. Linear mixed-effects models were performed using maternal identity as a random effect, while incubation environment, immune challenge, and non-correlated maternal variables were treated as fixed effects. When the random effect did not contribute significantly, simple linear models were used. For each model, residuals were tested to evaluate the suitability of the model and verify if the regression assumptions were met. For graphical representation relative expression of interest gene respect *r18s* values were plotted. All analyses were performed in R (Team, 2020) using the packages: car (Fox and Weisberg, 2019), coin (Hothorn et al., 2008), DHARMa (Hartig, 2022), emmeans (Lenth, 2023), lmtest (Zeileis and Hothorn, 2020), performance (Lüdecke et al., 2021) and stats (R Core Team, 2019). The graphics were created using the ggplot2 package (Wickham, 2016). Statistical significance was defined as $p \leq 0.05$.

4. Results

4.1 Maternal Parameters

Nesting females were in good physical condition at the time of examination, with no visible external lesions detected. Maternal body size and hemolytic capacity are summarized in Supplementary Table 1. Maternal hemolytic capacity was negatively liked with its body

mass (Supplementary Figure 1). No relationships were found between maternal parameters and clutch size (i.e. reproductive investment).

4.2 Incubation environment

The incubation environment markedly skewed sex ratios. Shaded hatcheries produced exclusively male hatchlings (100%), whereas unshaded hatcheries yielded predominantly females (73%) and 27% males.

Gene expression detectability varied across tissues and treatments (Table 2). In the liver, *il-1β* expression increased following LPS exposure in both incubation conditions (MEDIAN ± IQR. Unshaded PBS: 1.29 ± 1.41, LPS: 5.67 ± 5.71; Shaded PBS: 0.03 ± 0.41, LPS: 5.41 ± 2.40; $p \leq 0.001$), consistently exceeding expression levels in the PBS-unshaded group. In contrast, *il-6* expression was reduced in the PBS-shaded group (MEDIAN ± IQR. 0.03 ± 0.78) compared to all others (Unshaded PBS: 1.14 ± 3.40, LPS: 8.42 ± 7.68; Shaded LPS: 0.54 ± 5.58; $p = 0.005$). Antioxidant enzyme expression was generally downregulated in shaded-incubated hatchlings (MEDIAN ± IQR. *sod:* Shaded PBS: 0.23 ± 0.84, LPS: 0.13 ± 0.72 vs. Unshaded PBS: 0.97 ± 0.65, LPS: 1.18 ± 1.11, $p = 0.015$; *cat:* Shaded PBS: 0.31 ± 0.26, LPS: 0.27 ± 0.17 vs. Unshaded PBS: 0.50 ± 0.62, LPS: 0.81 ± 1.69, $p = 0.009$; MEAN ± SEM. *gr*: Shaded PBS: 0.33 ± 0.09, LPS: 0.10 ± 0.02 vs. Unshaded PBS: 1.61 ± 0.63, LPS: 0.77 ± 0.29, $p \leq 0.001$) with a major decrease in shaded-LPS hatchlings ($p = 0.034$). *Caspase-3* expression was likewise associated with unshaded hatcheries (MEDIAN ± IQR. Unshaded PBS: 1.12 ± 0.49, LPS: 0.75 ± 3.99 vs. Shaded PBS: 0.02 ± 2.33, LPS: 0.01 ± 1.84, $p = 0.026$). No statistical differences were found between groups in *tnf-α*, *hsp70* or *hsp90* (Figure 2, Table 2).

In the spleen, *cat* was responsive to incubation condition, showing increased expression in shaded-incubated hatchlings (MEDIAN ± IQR. Shaded PBS: 2.14 ± 1.28, LPS: 1.33 ± 0.67; Unshaded PBS: 0.86 ± 0.91, LPS: 0.65 ± 0.79; $p = 0.047$), while casp-*3* showed increased expression in unshaded-incubated hatchlings (MEDIAN ± IQR. Unshaded PBS: 1.15 ± 0.84, LPS: 0.76 ± 1.34; Shaded PBS: 0.53 ± 1.14, LPS: 0.39 ± 0.73; $p = 0.03$). No other spleen markers differed among groups (Figure 3, Table 2).

4.3 Maternal x environment x immune challenge effects

Linear mixed models and simple linear models were used to test if maternal traits could explain gene expression in challenged hatchlings incubated in unshaded and shaded hatcheries.

Maternal effects were identified only in the spleen. Mothers with greater SCW promoted the development of offspring with higher expression of *hsp90* (β = -0.35 ± 0.11, t = -3.16, p= 0.018), regardless of the incubation environment or immune challenge (Figure 4B, table 3). However, the maternal body mass (β = -0.68 ± 0.25, t = -2.69, p= 0.013) and hemolytic capacity (β = -0.04 ± 0.02, t = -2.18, p= 0.041) did interact with the incubation environment on hatchlings *hsp90* expression (Figure 4C, D, table 3). Specifically, greater maternal mass led to reduced *hsp90* expression in shaded-incubated hatchlings, whereas no association was found in those from unshaded hatcheries. Maternal hemolytic capacity was also negatively associated with hatchling's *hsp90* expression, with a stronger effect in unshaded-incubated offspring. In addition, interaction between maternal body mass and incubation environment had an effect on hatchlings' *cat* expression (Figure 4A, table 3), with heavier mothers promoting offspring with higher *cat* expression, but only in shaded-incubated hatchlings (β = -0.33 ± 0.14, t = 2.39, p= 0.025). Maternal CCL also had effect on hatchling's *cat* expression regardless of incubation environment (Supplementary figure 2), with larger mothers promoting offspring with lower *cat* expression (β = -0.60 ± 0.20, t = -3.01, p= 0.006). The immune challenge had no effect on these interactions. The models explained 54% and 51% of the variance in gene expression, respectively (Table 3).

5. Discussion

This study evaluated the effects of the incubation environment and its interaction with maternal traits on hepatic and splenic expression of proinflammatory cytokines, antioxidant enzymes, thermal-stress markers and a pro-apoptotic indicator in *Lepidochelys olivacea* hatchlings subjected to an immune challenge. The liver was the most responsive organ four hours after LPS exposure. Among the genes examined, only the proinflammatory cytokines *il-1β* and *il-6* responded to LPS challenge, whereas expression of *sod*, *cat*, *gr*

and *casp-3* in the liver was primarily shaped by the incubation environment. In the spleen, *cat* and *casp-3* expression were likewise associated with the incubation condition. In contrast, maternal effects were detected exclusively in the spleen, where maternal size and hemolytic capacity explained variation in *cat* and *hsp90* expression.

Developmental incubation conditions reorganize hepatic early-life immunophenotypes while preserving inducible *il-1β* inflammation

Immune responses to bacterial infection in reptiles, as in other vertebrates, rely on the coordinated upregulation of proinflammatory cytokines, yet these responses might be tightly constrained by energetic and metabolic costs, particularly during early life. In this study, hepatic *il-1β* expression increased in response to LPS in hatchlings from both incubation environments, suggesting that the capacity to mount an *il-1β*-driven inflammatory response is conserved despite contrasting developmental environments.

In contrast, *il-6*, *sod*, *cat*, *gr* and *casp-3* were strongly influenced by the incubation environment. Hatchlings incubated under shaded, cooler conditions exhibited increased *il-6* expression in response to LPS-challenge. Concurrently, these hatchlings showed reduced expression of *sod*, *cat*, *gr* and *casp-3* mRNA, independent of LPS exposure. In contrast, hatchlings from unshaded incubation conditions displayed higher expression levels of *il-6*, antioxidant and pro-apoptotic enzymes. From an immunometabolic perspective, these differences might imply alternative developmental strategies: shaded incubation may favor a punctual *il-6*-driven inflammatory response pathogen dependent constraining antioxidant buffering capacity during immune activation. In contrast, unshaded hatcheries may prime hatchlings toward a more inflammation-ready phenotype, potentially enhancing early pathogen defense but at the cost of increased oxidative and energetic burden. These interpretations are consistent with previous studies in the same species using a similar experimental design where hatchlings from shaded hatcheries exhibited increased hepatic expression of *hsp90*, *pi3k* and *rac1* at emergence, alongside a dampened inflammatory response to local immune challenge (García-Bucio et al., in review; García-Bucio et al., 2025). At the same time, offspring from shaded hatcheries display lower circulating hydrogen peroxide levels and reduced total antioxidant capacity

compared with unshaded counterparts (Robledo-Avila et al., 2023), supporting the notion that the incubation environment programs systemic redox and immune allocation.

Organ-specific responses further reinforce this framework. In both incubation environments, the liver mounted a proinflammatory response to LPS, paralleling observations in adult male toads (Floreste et al., 2023). Although often underappreciated as an immune organ, the liver plays a central role in coordinating acute-phase while maintaining tissue homeostasis (Ehlting et al., 2021; Kubes and Jenne, 2018). Because inflammatory activation and thermal stress can elevate reactive oxygen species, hepatic antioxidant defenses are essential to mitigate adverse effects and prevent apoptosis during immune activation (Ighodaro & Akinloye, 2018).

Taken together, these results support a model in which the incubation environment does not limit inducible expression of the core proinflammatory cytokine *il-1β*, but instead reprograms early life immunometabolic trade-offs that shape downstream inflammatory, antioxidant and pro-apoptotic responses. Under ongoing climate warming, such shifts in immunometabolic programming may have important implications for hatchling resilience, influencing how individuals balance pathogen defense against the physiological costs of immune activation in increasingly variable and stressful environments.

Maternal–environment interactions shape splenic immune trajectories in sea turtle hatchlings

Maternal effects were detected exclusively in the spleen, supporting the hypothesis that maternal inputs primarily influence immune development rather than immediate responsiveness, as proposed by recent theoretical and empirical work on context-dependent maternal effects (Fernandes and Lim, 2024; Kuijper and Hoyle, 2015). The maternal traits identified in this study have previously been shown to influence hatchlings' immune variables (Chávez-Salazar et al., 2025), reinforcing the consistency and functional relevance of maternal immune programming across studies.

In contrast, negative associations between maternal body mass and hemolytic capacity with offspring *hsp90* expression and a positive association between maternal straight

carapace width and offspring *hsp90* expression and maternal body mass with offspring *cat* expression point to trade-offs in antioxidant programming and thermal-stress programming. Such trade-offs may be adaptive for hatchlings developing under shaded, cooler incubation conditions, where thermal stress is reduced. However, under warmer and more stressful incubation environments, these maternally mediated reductions in protective pathways could increase vulnerability, highlighting the context dependence of maternal effects.

Among stress-related pathways, *hsp90* emerges as a key mediator of maternal-environment interactions. This chaperone has been proposed as a mechanism facilitating rapid adaptive responses when maternal cues reliably predict offspring environments, particularly under abrupt environmental change (Kim and Morales, 2025; Kuijper and Hoyle, 2015). Consistent with this role, previous studies in *Caretta caretta* demonstrate that offspring *hsp90* expression is sensitive to maternal environment and inherited immune signals (Tedeschi et al., 2015; Tedeschi et al., 2016). Similarly, in *L. olivacea*, hatchlings incubated under shaded conditions show elevated *hsp90* expression at nest emergence, suggesting that *hsp90* operates at the intersection of maternal programming and thermal developmental plasticity.

The spleen is a central secondary lymphoid organ involved in leukocyte maturation, cytokine production and immune education, especially during early life (Nash and Ryan, 2023; Zimmerman et al., 2010). In reptiles, the spleen plays a dominant role in humoral responses (Zimmerman et al., 2010). Previous studies in *L. olivacea* have shown increased splenic immune gene expression at nest emergence in hatchlings incubated in shaded hatcheries, consistent with environmental immune priming. In the present study, however, the spleen exhibited a limited transcriptional response to the acute LPS challenge and incubation environment, suggesting that splenic activation may occur outside the temporal window examined or follow a different activation trajectory than hepatic responses.

Taken together, these findings indicate that maternal immune programming operates through a dual mechanism: core inflammatory competence is canalized through maternal effects, whereas immunometabolic and stress-protective pathways remain

developmentally plastic and maternally tunable. Such differential sensitivity may critically shape offspring resilience under ongoing climate warming, where increasing thermal variability could constrain the adaptive value of maternally mediated immune trade-offs.

6. Conclusions

Incubation in unshaded nests appears to prepare hatchlings for a more stressful environment through an increased antioxidant expression, thus promoting a "set point" of the antioxidant system and some pro-inflammatory markers. These immuno-oxidative shifts may be advantageous if the post-hatching environment is stressful, but may also have energetic costs or affect processes such as apoptosis and repair (Tobler et al., 2015). In contrast, hatchlings incubated in the shaded hatcheries may be more vulnerable to an immune challenge because the antioxidant capacity of *gr* decreases when exposed to LPS, which could translate into greater oxidative damage during infections. However, because shaded-incubated hatchlings might not be able to produce autonomic fever, it would be advisable to evaluate whether the response of hatchlings incubated in shaded environments and subjected to an immune challenge improves their immune response when they are allowed to unleash a behavioral fever.

Acknowledgements

The authors thank the staff of the “Centro Ecoturístico Escobilla” hatchery for their valuable technical assistance.

Funding

This work was supported by the Coordinación de la Investigación Científica-UMSNH to EMH. SNCS is a SECIHTI Ph.D. fellow (grant number: 817553, SNCS).

Conflict of interest

This work is not under consideration for publication elsewhere, and no conflicts of interest are associated with its publication.

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Table 1. Primers and melting temperatures (Tm) for RT-qPCR mRNA analysis.

GENE	PRIMER SEQUENCE FOR PCR	Tm (°C)	REFERENCE
r18s	Forward: GCTAATACATGCCGACGAG Reverse: GGCCCGAGGTTATCTAGAG	60	Tedeschi et al., 2015
tnf-α	Forward: TGAGCACCGAAAGTCTGGTC Reverse: TCTGAAATGCAGCAGAGCGA	60	Cocci et al., 2022
il-1β	Forward: TGCAGTTCACAGAGCAGACC Reverse: GACCGGAAACCTCATGGAG	60	Rayl et al., 2019
il-6	Forward: CAGTGATCATGCCAGACCCA Reverse: TCGAACAGCCCTCACAGTTT	60	Cocci et al., 2022
sod	Forward: TGGATGTACCAGTGCAGGTG Reverse: CAATCACATTGCCAAGATCG	55	Cortés-Gómez et al., 2018
cat	Forward: GATCAGCCCAATTTGAAGGA Reverse: TCTCACACAGTCTTTGGCGTTC	55	Cortés-Gómez et al., 2018
gr	Forward: TGCACTCAGGAACTGGAGAA Reverse: CCTCCAACAATCCAAGAGG	60	Cortés-Gómez et al., 2018
hsp70	Forward: TCTCCGTACAGCTTGTGAAC Reverse: CCACGGAACAGATCAGC	60	Tedeschi et al., 2015
hsp90	Forward GGATACTGGCATAGGGATG Reverse CAACACCAAACTGACCAATC	57	Tedeschi et al., 2015
casp-3	Forward: ATCCTCCCTGGATAAATGTGC Reverse: ATCTGAGTGGAGATGGCCTG	60	Accession number NC 057852.1

Table 2. Summary of tests evaluating the effect of the incubation condition (unshaded vs. shaded), challenge (PBS vs. LPS) and its interaction on liver and spleen gene expression in *Lepidochelys olivacea* hatchlings.

Gene	LIVER Incubation Condition and Challenge				Test statistic $_{(d.f.)}$, *p*		
	U PBS	U LSP	S PBS	S LPS	Incubation	Challenge	ExC
	Mean ± SEM						
gr	1.61 ± 0.63	0.77 ± 0.29	0.33 ± 0.09	0.10 ± 0.02	-4.48, **<0.001**	-2.20, **0.03**	0.40, 0.69
hsp70	1.59 ± 0.50	3.43 ± 1.07	3.27 ± 1.91	2.32 ± 1.16	-0.14 $_{(27)}$, 0.89	1.38 $_{(27)}$, 0.18	-0.73 $_{(27)}$, 0.47
hsp90	1.28 ± 0.21	1.30 ± 0.34	1.04 ± 0.52	1.33 ± 0.54	-1.10 $_{(27)}$, 0.28	1.31 $_{(27)}$, 0.20	-0.94 $_{(27)}$, 0.36
	Median ± IQR						
tnf-α	1.65 ± 5.47	1.01 ± 4.15	0.02 ± 1.24	0.05 ± 2.82	-1.98, 0.06	0.32, 0.75	-0.12, 0.90
il-1β	1.29 ± 1.41	5.67 ± 5.71	0.03 ± 0.41	5.41 ± 2.40	-1.40, 0.17	5.91, **<0.001**	-1.58, 0.12
il-6	1.14 ± 3.40	8.42 ± 7.68	0.03 ± 0.78	0.54 ± 5.58	-1.67, 0.10	2.97, **0.005**	-0.87, 0.39
sod	0.97 ± 0.65	1.18 ± 1.11	0.23 ± 0.84	0.13 ± 0.72	-2.57, **0.015**	-0.39, 0.70	0.22, 0.83
cat	0.50 ± 0.62	0.81 ± 1.69	0.31 ± 0.26	0.27 ± 0.17	-2.81 $_{(24.70)}$, **0.009**	-0.98 $_{(25.15)}$, 0.34	0.59 $_{(25.54)}$, 0.56
casp-3	1.12 ± 0.49	0.75 ± 3.99	0.02 ± 2.33	0.01 ± 1.84	-2.46 $_{(15.54)}$, **0.026**	0.32 $_{(16.55)}$, 0.75	0.11 $_{(16.85)}$, 0.91
	SPLEEN						
	Mean ± SEM						
il-1β	1.56 ± 0.68	2.54 ± 0.68	1.02 ± 0.24	2.30 ± 0.60	0.09 $_{(20.49)}$, 0.93	1.99 $_{(21.20)}$, 0.06	-0.42$_{(23.13)}$, 0.68
il-6	1.31 ± 0.29	1.03 ± 0.30	0.53 ± 0.14	0.69 ± 0.29	-0.40 $_{(25.21)}$, 0.69	1.40 $_{(25.56)}$, 0.18	-1.64 $_{(25.21)}$, 0.11
sod	5.40 ± 3.17	4.84 ± 4.38	4.36 ± 2.37	2.80 ± 2.15	0.23 $_{(27)}$, 0.82	-0.40 $_{(27)}$, 0.70	0.15 $_{(27)}$, 0.88
	Median ± IQR						
tnf-α	1.17 ± 0.27	1.43 ± 1.18	1.06 ± 0.59	1.05 ± 0.86	-0.42 $_{(12)}$, 0.68	-0.56 $_{(12)}$, 0.59	0.19 $_{(12)}$, 0.85
cat	0.86 ± 0.91	0.65 ± 0.79	2.14 ± 1.28	1.33 ± 0.67	2.07, **0.047**	-0.69, 0.49	0.03, 0.97
hsp70	0.98 ± 0.42	0.83 ± 0.76	1.22 ± 0.98	0.46 ± 0.84	-1.00, 0.32	-0.90, 0.38	0.00, 1.00
hsp90	0.92 ± 0.65	0.94 ± 0.80	0.81 ± 0.81	0.39 ± 0.90	-0.61, 0.54	0.06, 0.95	-0.78, 0.44
casp-3	1.15 ± 0.84	0.76 ± 1.34	0.53 ± 1.14	0.39 ± 0.73	-2.30 $_{(27)}$, **0.03**	-1.20 $_{(27)}$, 0.24	0.61 $_{(27)}$, 0.54

d.f., degrees of freedom; *p*, test p-value. Bold values denote significant effects at $p \leq 0.05$.

Table 3. Summary of the models explaining mRNA expression of stress-related and innate immune response molecules in the spleen of sea turtle hatchlings incubated under unshaded and shaded hatcheries in relation to maternal variables.

SPLEEN	β	t (d.f)	*p*	R^2
cat (linear)		(7, 25)		0.51
Intercept	-8.61	-0.71	0.486	
Incubation condition	13.66	0.83	0.415	
mSCW	-0.16	-1.53	0.140	
mCCL	0.60	3.01	**0.006**	
mMass	-0.21	-2.14	**0.042**	
mSCW: Incubation condition	0.20	1.21	0.238	
mCCL: Incubation condition	-0.52	-1.72	0.098	
mMass: Incubation condition	0.33	2.39	**0.025**	
Hsp90 (mixed)				
Intercept	35.49	2.02 (6.31)	0.088	0.54
Incubation condition	-22.37	-1.39 (22)	0.179	
mSCW	-0.35	-3.16 (6.31)	**0.018**	
mCCL	-0.55	-1.66 (6.31)	0.145	
mMass	0.82	2.97 (6.31)	**0.024**	
mHC	0.07	3.37 (6.31)	**0.014**	
mSCW: Incubation condition	0.19	1.86 (22)	0.076	
mCCL: Incubation condition	0.53	1.77 (22)	0.090	
mMass: Incubation condition	-0.68	-2.69 (22)	**0.013**	
mHC: Incubation condition	-0.04	-2.18 (22)	**0.041**	

β, regression coefficient; d.f., degrees of freedom; t, Student t-test indicator; *p*, Satterthwaite´s *p*-value; R^2, marginal coefficient of determination. Bold values denote significant effects at $p \leq 0.05$. Note that all the mixed effects models include an interaction with maternal variables

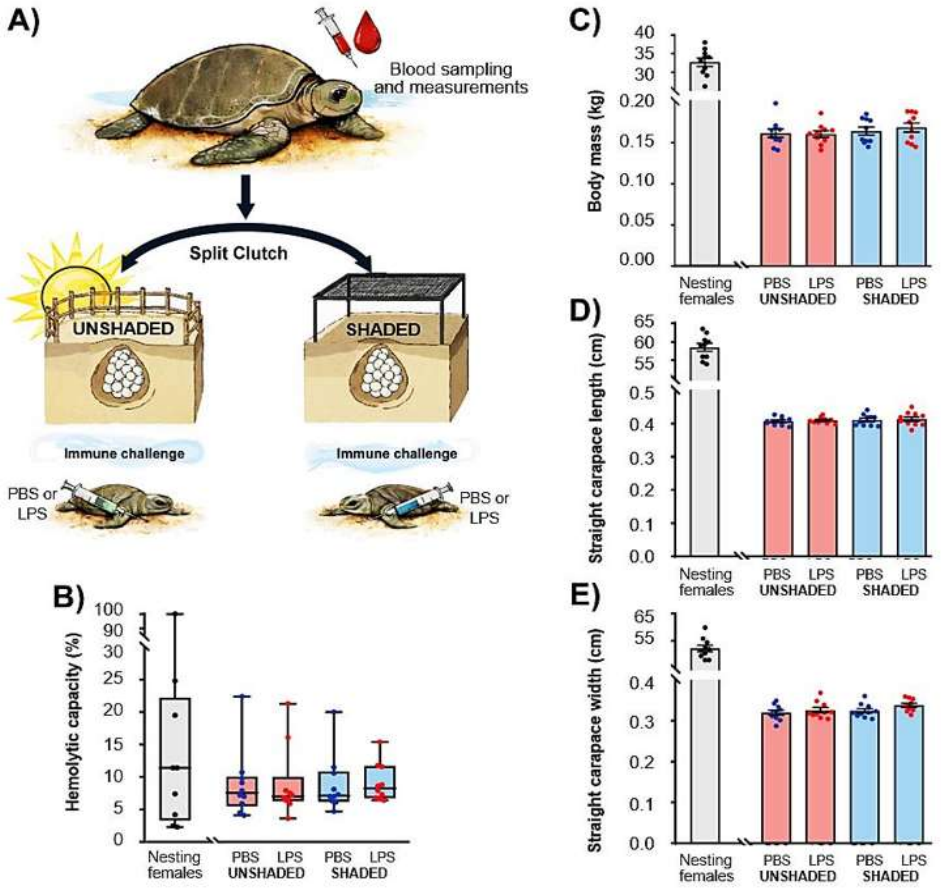


Figure 1. Experimental design scheme. A) The first level represents the obtention of morphological measurements and blood samples from nesting females. In the second level split clutch design is shown. One half of each clutch was reburied in an unshaded hatchery and the other half in a shaded hatchery. The lower level represents two hatchlings by nest from unshaded and shaded hatchery receiving an intraperitoneal injection with PBS or LPS. B) Graph of the hemolytic capacity percentage of mothers (gray bar) and hatchlings injected with PBS and LPS from unshaded (pink bars) and shaded (blue bars) conditions. C) Graph of the body mass of mothers (gray bar) and hatchlings injected with PBS and LPS from unshaded (pink bars) and shaded (blue bars) conditions, D) Graph of the length of mothers (gray bar) and hatchlings injected with PBS and LPS from unshaded (pink bars) and shaded (blue bars) conditions and E) Graph of the width of mothers (gray bar) and hatchlings injected with PBS and LPS from unshaded (pink bars) and shaded (blue bars) conditions.

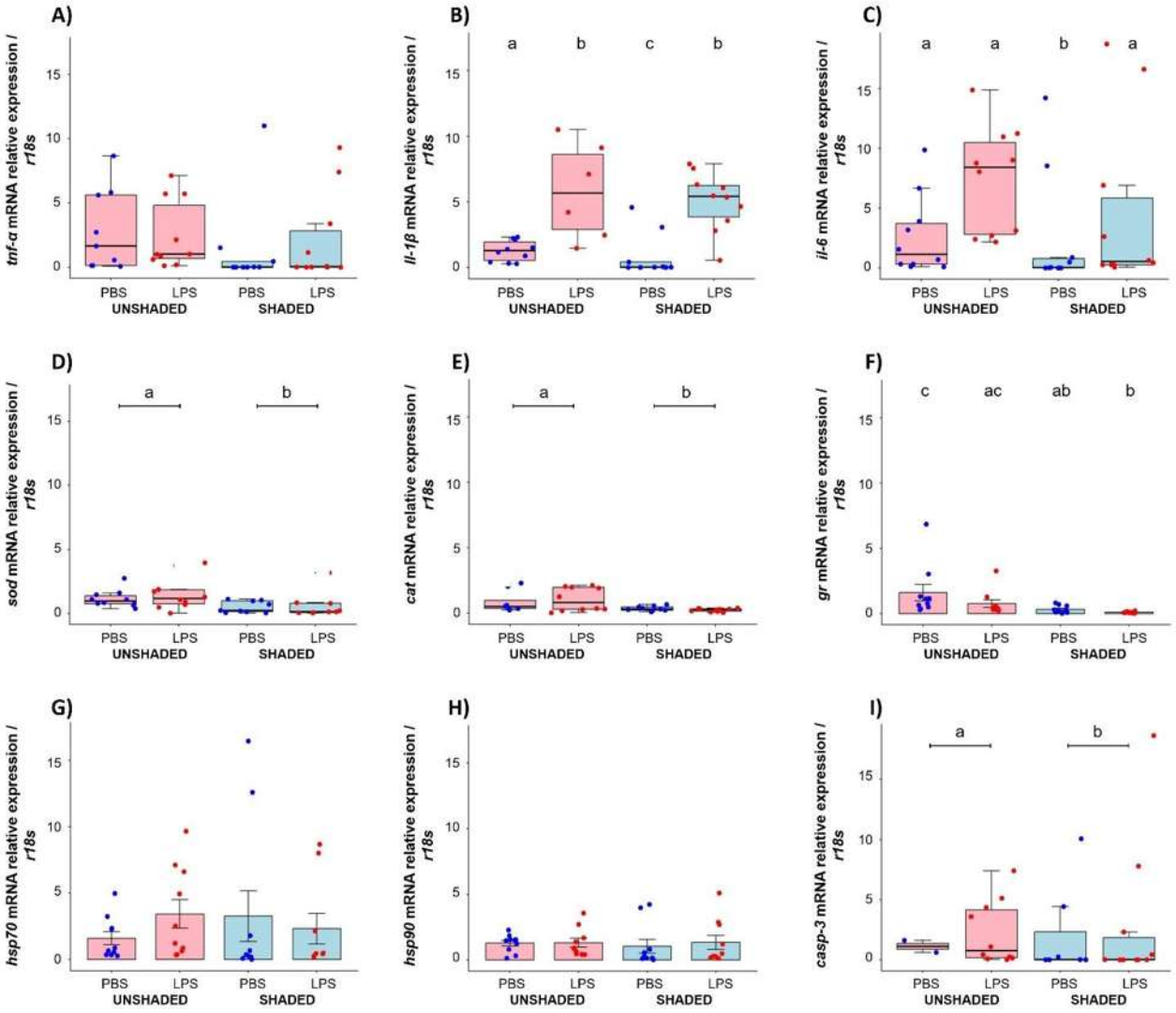


Figure 2. Expression in liver of A) *tnf-α*, B) *il-1β*, C) *il-6*, D) *sod*, E) *cat*, F) *gr*, G) *hsp70*, H) *hsp90* and I) *casp-3*. Groups in pink represent unshaded and light blue the shaded incubation environment. Points in blue are hatchlings inoculated with PBS and red points represent hatchlings challenged with LPS. Lowercase letters indicate differences between groups with $p \leq 0.05$.

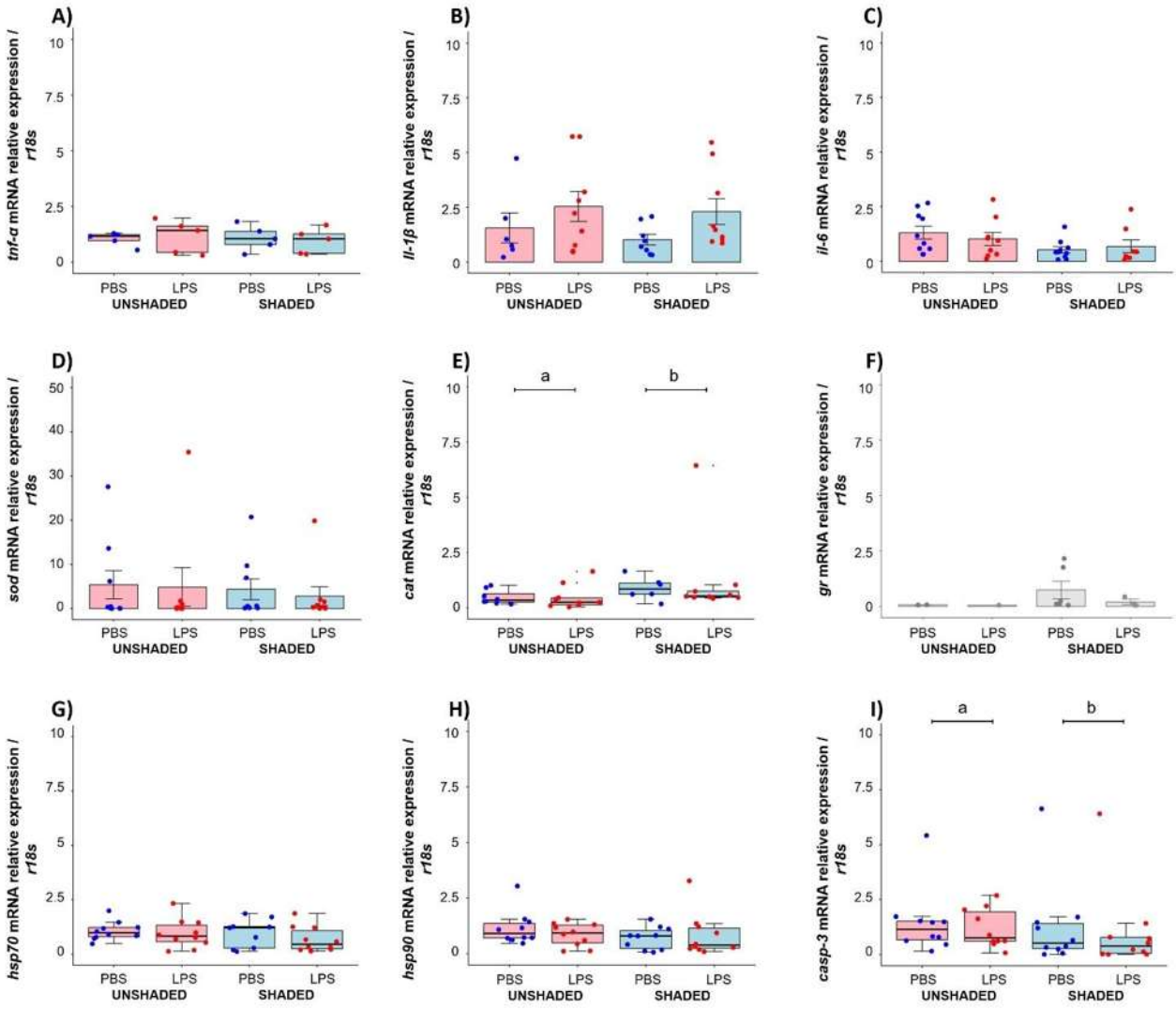


Figure 3. Expression in spleen of A) *tnf-α*, B) *il-1β*, C) *il-6*, D) *sod*, E) *cat*, F) *gr*, G) *hsp70*, H) *hsp90* and I) *casp-3*. In *gr* case, the graph is represented in gray because more than 50% of the CTs were above the detection threshold and were not considered for statistical analysis. For the rest of the graphs, groups in pink represent unshaded and light blue the shaded incubation environment. Points in blue are hatchlings inoculated with PBS and red points represent hatchlings challenged with LPS. Lowercase letters indicate differences between groups with $p \leq 0.05$.

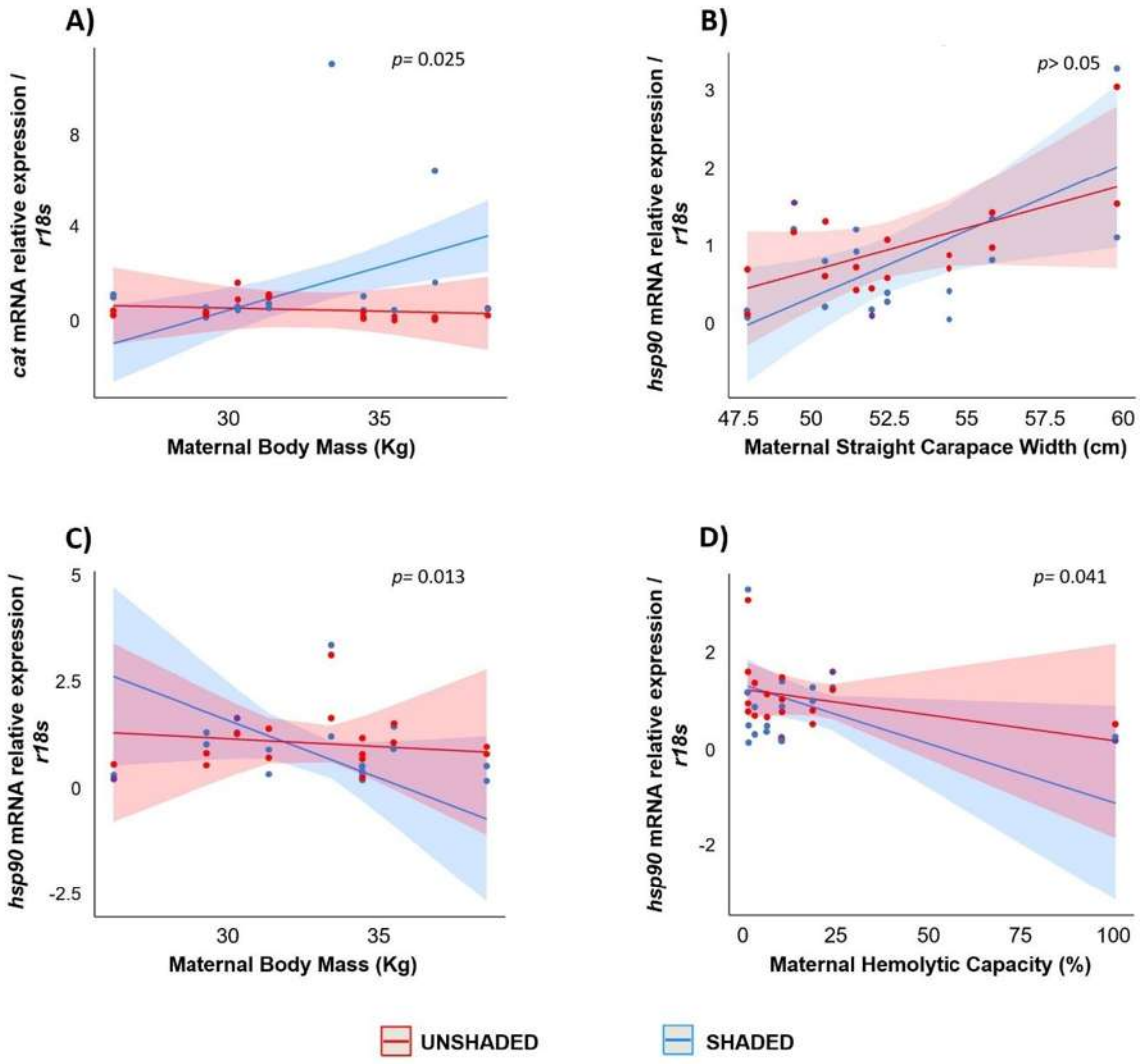
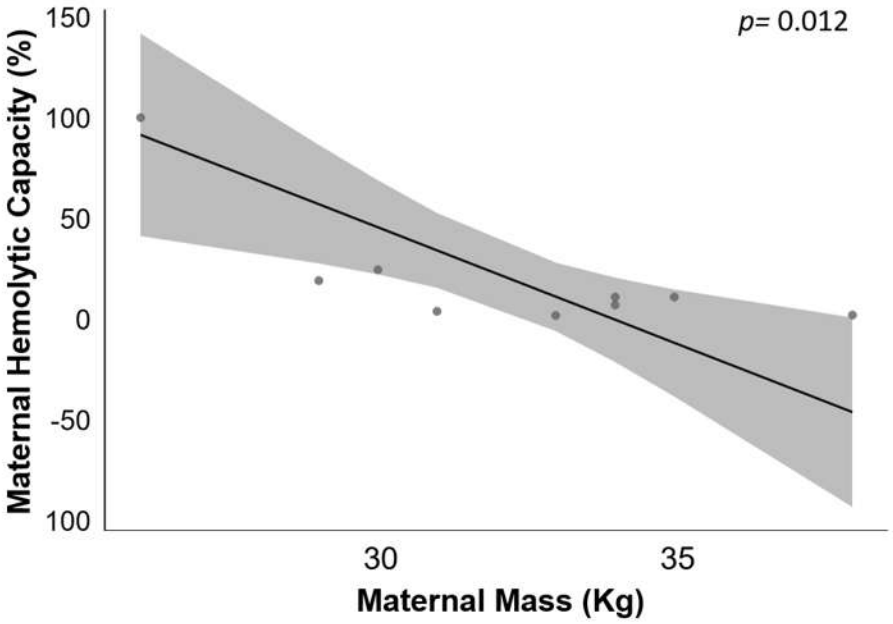


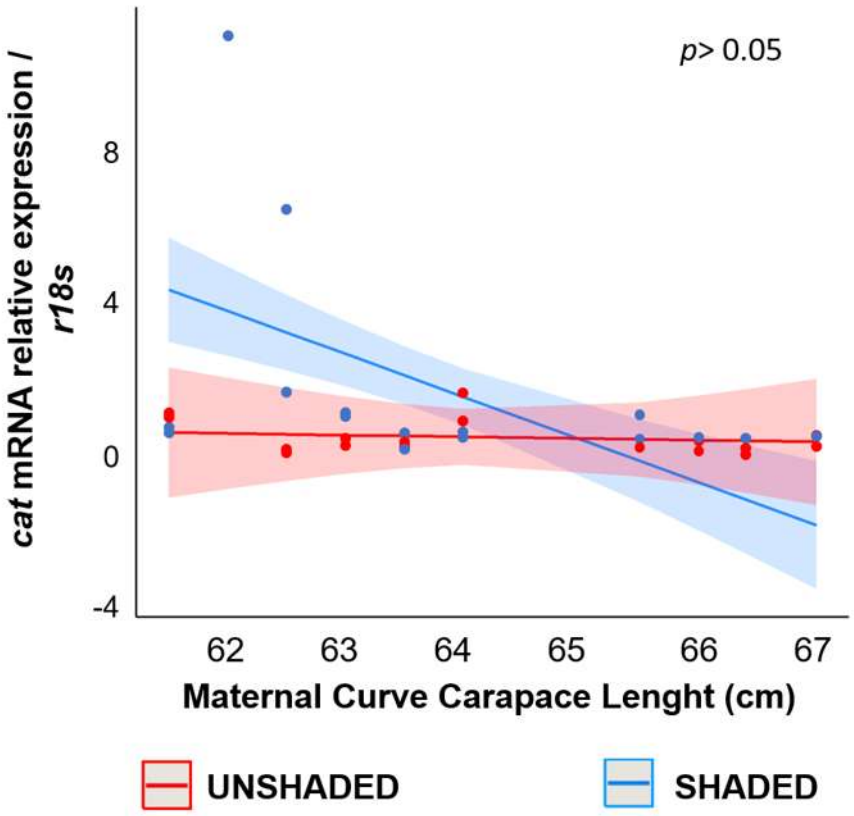
Figure 4. Graphs depict the linear relationships between maternal parameters and hatchling splenic expression of A) maternal body mass (Kg) and hatchlings *cat* expression, B) maternal straight carapace width (cm) and hatchlings *hsp90* expression, C) maternal body mass (Kg) and hatchlings *hsp90* expression and D) maternal hemolytic capacity (%) and hatchlings *hsp90* expression. Red points from each graph represent hatchlings from unshaded and blue points represent hatchlings from shaded. Lines indicate the estimated relationship from linear mixed models, with 95% confidence intervals shown as pink shading for unshaded and light blue shading for shaded incubation environments. Corresponding p-values for each model provide a difference between group slopes. All the relations had statistical significance regardless of the difference between groups' slopes.

Supplementary Table 1. Summary of body size, hematological and maternal investment indicators measured in nesting females.

Body size	**Mean ± SD**	**Range**
Straight carapace length (SCL, cm)	58.52 ± 3.28	54 - 63.5
Straight carapace width (SCW, cm)	52.23 ± 3.72	48 – 59.9
Curve carapace length (CCL, cm)	64.14 ± 1.96	61.5- 67
Curve carapace width (CCW, cm)	67.93 ± 3.21	62-72.5
Mass (kg)	32.63 ± 3.63	26-38
Hemolytic capacity (%)	20.39 ± 30.82	2.3-100
Clutch size	89.20 ± 13.02	74-107



Supplementary Figure 1. Graphic of the linear relationships between maternal body mass (x axis) and hemolytic capacity (y axis). Gray points from the graph represent sampled females. Line indicates the estimated relationship from linear mixed models, with 95% confidence intervals shown as gray shading. *p*-value for the model is provided.



Supplementary Figure 2. Graphic of the linear relationship between maternal curve carapace length (cm) and hatchlings *cat* expression. Red points represent hatchlings from unshaded and blue points represent hatchlings from shaded. Lines indicate the estimated relationship from linear mixed models, with 95% confidence intervals shown as pink shading for unshaded and light blue shading for shaded incubation environments. The *p*-value of the model provides the difference between group slopes. The relation had statistical significance regardless of the difference between groups' slopes.

VI. DISCUSIÓN GENERAL

Este estudio evaluó desde un enfoque ecofisiológico, el efecto del estado físico e inmunitario materno (Capítulo 1 y 2) y su interacción con ambientes de incubación contrastantes (sin sombra y sombra; Capítulo 2) sobre de la respuesta inmunitaria innata de neonatos de tortuga golfina *Lepidochelys olivacea* sometidas a un reto inmunitario

simulado con LPS bacteriano. El efecto materno fue evaluado mediante la talla corporal y la capacidad lítica contra eritrocitos de borrego, mientras la respuesta inmunitaria de las crías frente al reto con LPS fue determinada a través de la capacidad lítica (Capítulo 1) y la expresión de marcadores proinflamatorios, enzimas antioxidantes, proteínas de choque térmico y el marcador proapoptótico *casp-3* en hígado y bazo (Capítulo 2).

Los resultados del Capítulo 1 indican que las hembras con mayor capacidad hemolítica y mayor masa corporal favorecen una mayor capacidad hemolítica en sus crías, mientras que se observó una relación opuesta con la longitud recta del caparazón materno. Es posible que la exposición previa de la madre a diversos patógenos favorezca la preparación inmunitaria de sus crías (Schumacher et al., 1999). Por lo que respecta a la masa corporal, se ha propuesto que puede reflejar la disponibilidad de recursos energéticos (Bernardo, 1996; Houston et al., 2007). Debido a que la capacidad hemolítica constituye un mecanismo de defensa esencial, es posible que las hembras más pesadas inviertan un mayor suministro de recursos para favorecer este aspecto en sus crías. No obstante, debido a que la energía disponible en los organismos es finita, se deben hacer negociaciones entre el aporte de recursos inmunitarios que destinan entre diferentes aspectos (Bernardo,1996; Charnov y Krebs, 1974).

Debido a que las tortugas marinas carecen de cuidado parental físico, la transferencia materna de moléculas inmunitarias podría servir como estrategia adaptativa para mejorar los mecanismos de defensa de sus crías durante las etapas tempranas de la vida (Grindstaff et al., 2003; Hasselquist y Nilsson 2012; Virgin et al., 2022). Aunque en el presente estudio no se identificó la naturaleza de las moléculas involucradas, es posible que las hembras transfieran a sus crías, a través del vitelo, componentes del sistema del complemento o péptidos antimicrobianos con actividad lítica, como ha sido descrito en mamíferos (Dutta y Das, 2016; Xu et al., 2024), o bien reservas energéticas que faciliten la síntesis de moléculas hemolíticas en la descendencia, como ha sido sugerido en aves y reptiles (Brown y Shine, 2016; Hasselquist y Nilsson, 2012; Karell et al., 2008; Virgin et al., 2022). Una de las moléculas cuya transferencia podría favorecer el entrenamiento térmico de la madre a las crías es la *hsp90*. Estudios previos en *Caretta caretta* han

demostrado que la expresión de *hsp90* es sensible al ambiente experimentado y su expresión en las crías puede estar influenciada por las condiciones experimentadas por la madre y por señales inmunitarias heredadas (Tedeschi et al. 2015; Tedeschi et al. 2016). Esta influencia materna podría facilitar la adaptación de sus crías ante ambientes cambiantes. El hecho que en este estudio (Capítulo 2) la expresión de *hsp90* en el bazo de las crías sea explicada por la talla corporal y la capacidad hemolítica materna apoyan el papel de *hsp90* como molécula relevante de la transferencia materna en la programación temprana de mecanismos de defensa celular.

Por otra parte, los resultados del Capítulo 2 de este estudio muestran la función hepática y esplénica frente a un reto bacteriano simulado y su relación con dos ambientes contrastantes de incubación. Uno de los hallazgos más relevantes es la respuesta de la citocina proinflamatoria *il-1β*, cuya expresión incrementó en el hígado de las crías provenientes de ambas condiciones de incubación, sin diferencias significativas entre los grupos LPS. De manera similar, la *il-6* tuvo un incremento significativo entre el grupo PBS y el LPS únicamente en los viveros sombreados, y esta expresión no fue diferente de ambos grupos de la condición sin sombra, lo que sugiere que las crías incubadas en nidos sin sombra presentan un estado proinflamatorio basal, mientras que las de viveros sombreados inflaman únicamente en respuesta al reto inmunitario. Otro resultado relevante es la expresión diferencial de enzimas antioxidantes y la enzima proapoptótica entre condiciones de incubación. De manera general, las cuatro enzimas estudiadas mantuvieron una menor expresión en el hígado de las crías incubadas en condiciones de sombra con respecto a los ambientes sin sombra, siendo el grupo retado con LPS en sombra el que presenta menor expresión de *gr.* Es posible que debido a que los viveros sombreados proporcionan temperaturas cercanas a las óptimas para la especie, las crías no requieren activar su defensa antioxidante, no obstante, ante un desafío inmunitario, la respuesta inflamatoria podría afectar sus mecanismos redox (Liu et al., 2026).

VII. CONCLUSIÓN

Los resultados obtenidos en este estudio sugieren que la respuesta inmunitaria innata de crías de *Lepidochelys olivacea* está determinada por rasgos maternos y su interacción con

el ambiente de incubación. La defensa inmunitaria de las crías estuvo favorecida en aquellas cuyas madres tuvieron un mayor tamaño y capacidad hemolítica, mientras que el ambiente de incubación modificó la respuesta inflamatoria y antioxidante frente a un reto inmunitario. Desde un contexto ecofisiológico y de conservación, nuestros hallazgos resaltan que la vulnerabilidad o resiliencia de las tortugas marinas en respuesta al cambio ambiental puede comenzar desde etapas del desarrollo embrionario por efecto de rasgos maternos en interacción con las condiciones ambientales experimentadas por las crías al interior del nido, particularmente la temperatura. Bajo el escenario del cambio climático, comprender estos mecanismos resulta de gran relevancia ya que puede contribuir al diseño de mejores estrategias de manejo en viveros que se aproximen más a los requerimientos fisiológicos de los individuos y promuevan el desarrollo de crías potencialmente más aptas para enfrentar los cambios ambientales.

VIII. PERSPECTIVAS Y RECOMENDACIONES

Debido a que trabajamos con una especie protegida, nuestro tamaño de muestra estuvo limitado y no se realizó una curva de la respuesta inmunitaria a diferentes horas. Futuros estudios podrían evaluar en otros puntos de tiempo post inoculación, la respuesta esplénica de crías retadas con LPS para identificar la actividad máxima de los marcadores inmunitarios utilizados en este estudio.

El presente estudio se centró en la evaluación de un conjunto acotado de marcadores de respuesta inmunitaria, lo que permite una aproximación inicial a los procesos involucrados. No obstante, resulta necesario incorporar indicadores complementarios que abarquen otras rutas funcionales para comprender con mayor profundidad las consecuencias biológicas de la expresión diferencial de genes observada tanto entre condiciones de incubación como en respuesta al desafío inmunitario con LPS.

Aunque los resultados obtenidos en esta investigación contribuyen a describir la cascada de señalización intracelular de la respuesta inmunitaria en las tortugas marinas, aún no se identifican bien cuáles vías se activan en respuesta al LPS y cuáles lo hacen en respuesta

al ambiente. La evaluación de marcadores adicionales involucrados en las diferentes vías podría ayudar a identificar mejor las rutas de señalización.

Otros aspectos que podrían considerarse en futuras investigaciones son análisis histológicos para evaluar la integridad tisular ante el reto con LPS, la elaboración de ensayos que midan el daño oxidante e incluso se podría evaluar la eficiencia inmunitaria frente a otros microorganismos.

La respuesta inmunitaria puede estar influenciada por el sexo de los individuos. Debido a que la determinación sexual en tortugas marinas depende de la temperatura, en estudios ecofisiológicos como este, es muy complicado obtener machos y hembras entre ambientes. Sin embargo, estudios posteriores podrían experimentar la manipulación hormonal embrionaria que permita obtener un equilibrio sexual entre ambientes y evaluar la respuesta inmunitaria de las crías ante un reto inmunitario.

IX. LITERATURA CITADA

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Efecto de la inmunocompetencia materna y del ambiente de incubación sobre la respuesta inmunitaria innata de crías de L...

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