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Adaptaciones fisiológicas al estrés: Estado redox, inmunocompetencia y glucocorticoides en dos especies de murciélagos del Género Myotis

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Lic. Ivonne Ramírez Wence
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P r e s e n t e

Me permito informar a usted que en la reunión del Subcomité por Campo de Conocimiento de Ecología y Manejo Integral de Ecosistemas del Posgrado en Ciencias Biológicas, celebrada el día 16 de abril de 2018, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **HERNÁNDEZ ARCIGA ULALUME** con número de cuenta **511021321** con la tesis titulada: **"Adaptaciones fisiológicas al estrés: Estado redox, inmunocompetencia y glucocorticoides en dos especies de murciélagos del Género *Myotis*"**, realizada bajo la dirección del **DR. LUIS GERARDO HERRERA MONTALVO:**

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COORDINADOR DEL PROGRAMA

c.c.p. Expediente del (la) interesado (a).

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But were stopped by the door of a tomb—
By the door of a legended tomb;
And I said—"What is written, sweet sister,
On the door of this legended tomb?"
She replied—"Ulalume—Ulalume—
'Tis the vault of thy lost Ulalume!"

Edgar Allan Poe, 1847

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RESUMEN

Entender las capacidades fisiológicas de los organismos para lidiar con diferentes factores estresantes derivados del ambiente, se ha convertido en un foco de interés para ecólogos y fisiólogos. El objetivo de esta tesis fue determinar las estrategias fisiológicas utilizados por los animales adaptados a ambientes potencialmente estresantes, y sus respuestas ante estímulos estresores, mediante la medición de diferentes marcadores de estrés como cambios en el estado redox, en el que se midieron antioxidantes enzimáticos (SOD, CAT y GPx) y la concentración de carbonilos (como medida de daño oxidativo) en sangre. También se midieron los metabolitos de glucocorticoides [FCM] en heces, y para determinar la inmunocompetencia se midió actividad bactericida (BA) en plasma (respuesta humoral) y reto por fitohemaglutinina (respuesta inflamatoria).

El objetivo del primer capitulo titulado "**Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat *Myotis vivesi***", fue A) determinar el estado redox y la variación basal inmune estacional en Myotis (*Myotis vivesi*) y B) determinar el efecto que tiene un estímulo de estrés agudo sobre la inmunocompetencia y el estado redox a lo largo del año. El estrés agudo se estimuló restringiendo el movimiento de los animales durante 6 y 12 h. Se encontró que los niveles basales de antioxidantes de esta especie variaron a lo largo del año, siendo más altos en otoño y más bajos en verano e inicios/finales del invierno. Por su parte, los niveles de daño a proteínas tuvieron un patrón inverso al de los antioxidantes. Después de 6h de estrés los niveles de antioxidantes y BA aumentaron durante el verano y finales de invierno, sin evidencia de daño a proteínas, sin embargo después de 12 h el daño a las proteínas se empezó a acumular.

Los objetivos del segundo capitulo titulado "**Glucocorticoids seasonal variations and its relation to immunocompetence and oxidative burst in the fishing bat *Myotis vivesi***",

fueron determinar A) la variación de FCM en murciélago *Myotis vivesi*, a lo largo del año, y la intensidad del cambió en su concentración después de un estrés agudo, y B) relacionarlo con los cambios en la respuesta inmune y estado redox de los organismos. Se encontró que los niveles basales de FCM fueron menores a finales del invierno en comparación de las demás estacones, y después de 12 h de estrés aumentaron en todas las estaciones, siendo mayores en verano y principios de invierno; después de 24 h de estrés los niveles de FCM aumentaron homogéneamente en todas las estaciones. También se encontró que en condiciones basales, a mayor niveles FCM menor respuesta inflamatoria; después de 12 h de estrés aumentó la respuesta inflamatoria, pero después de 24 h disminuyó. La respuesta humoral estuvo relacionada siempre positivamente con los niveles de FCM, tanto en condiciones basales como de estrés.

El objetivo del tercer capitulo titulado "**Changes in redox state in different tissues after interruption of hibernation in *Myotis velifer*"** fue determinar cambios en el estado redox y daño oxidativo al interrumpir el ciclo de hibernación en *Myotis velifer*. Se encontró que la actividad enzimática de los antioxidantes en la sangre aumentó al salir del torpor y disminuyó en cerebro y pulmones, sin presentar cambios en hígado y tejido adiposo blanco (TAB). No se encontró daño a proteínas en ningún tejido, e incluso disminuyó en la sangre. Esto sugiere que la explosión oxidativa podría estar ocurriendo antes de que se alcance una temperatura corporal eutérmica y que existe una reposición de antioxidantes a los órganos. Además, se encontró que la actividad antioxidante estaba directamente relacionada con la prevención de daño oxidativo en algunos casos (sangre, corazón y TAB). En conclusión, en este trabajo se encontró que los organismos adaptados a vivir en condiciones adversas cuentan con los mecanismos fisiológicos necesarios que los hace resistentes a eventos de estrés; sin embargo cuando el estrés se prolonga o coincide con otras actividades energéticamente costosas (i.e la lactancia) pueden sobrepasar las defensas de los organismos.

ABSTRACT

Understanding the physiological capacities of organisms against different stress factors derived from the environment has become a focus of interest for ecologists and physiologists. The objective of this thesis was to determine the physiological strategies of animals adapted to adverse environments, and their response to stress stimuli, through the measurement of different stress markers, such as the changes in the redox state, by measuring enzymatic antioxidants (SOD, CAT and GPx), and the concentration of carbonyls (as a measure of oxidative damage) in blood. Fecal cortisol metabolites (FCM) were also measured. Bactericidal activity (BA) (humoral response), and Phytohemaglutanin challenge (inflammatory response) were determined as immunocompetence indicators.

The first chapter "**Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat *Myotis vivesi***", objective was to A) determine seasonal variations in basal redox state and immunocompetence in the fishing bat *Myotis vivesi*, and B) determine the effect that a stress stimuli has over the redox state and the immunocompetence through the seasons. Restricting the animal's movement for 6 and 12 h stimulated the acute stress stimulus. It was found that the basal levels of antioxidants of this species varied throughout the year. They were higher in autumn and lower in summer and early/late winter. On the other hand, protein damage levels had a reverse pattern to that of antioxidants. Antioxidants and BA levels increased during summer and late winter, after 6h of stress without evidence of protein damage. However, after 12 h the protein damage began to accumulate.

The objectives of the second chapter "**Glucocorticoids seasonal variations and its relation to immunocompetence and oxidative burst in the fishing bat *Myotis vivesi*"**, were to determine A) the variation of FCM in *Myotis vivesi* bat, throughout the year, and the change

intensity in concentration of FCM after an acute stress; B) to determine its relationship with changes in the organismla immune response and redox state. It was found that the basal levels of FCM were lower at the end of winter compared to the other states, and after 12 h of stress they increased in all seasons, being higher in summer and early winter; after 24 h of stress, FCM levels increased homogeneously in all stations. It was also found that in basal conditions, at higher FCM levels, less inflammatory response, after 12 h of stress the cellular immune response increased, but 24 h low. The humoral response was always positively related to FCM levels in both baseline and stress conditions.

The objectives of the third chapter "**Changes in redox state in different tissues after interruption of hibernation in *Myotis velifer"*** were to determine the changes in the redox state and oxidative damage by interrupting the hibernation cycle in Myotis velifer. The enzymatic activity of antioxidants increased in blood and decreased in brain and lungs after arousal, with no changes in the liver and white adipose tissue (WAT). No protein damage was found either in any tissue. This means that the oxidative burst could be occurring before the bats reached a euthermic body temperature and that there was a replacement and redistribution of antioxidants to the organs. In addition, there was evidence that antioxidant activity was directly related to the prevention of oxidative damage in some cases (blood, heart and WAT). In conclusion, in this work we found that the organisms adapted to live in adverse conditions have the necessary physiological adaptations that make them resistant to stress; however, when stress is prolonged or coincides with other energy-costly activities (i.e, lactation) they may exceed the defenses of the organisms.

INTRODUCCIÓN GENERAL

El concepto de estrés.

El concepto de estrés empleado dentro de las investigaciones fisiológicas y biomédicas fue introducido por Selye (1950); desde entonces ha sido continuo objeto de debate científico. Originalmente, el concepto de estrés se definió como: la respuesta inespecífica del cuerpo ante cualquier estímulo nocivo. Más adelante el concepto se re-definió al hacer la distinción entre "*estresor*" y "*respuesta al estrés*". Un "*factor estresante*" se considera como un estímulo que amenaza la homeostasis, mientras que la "*respuesta al estrés*" es la reacción del organismo dirigida a recuperar la homeostasis (1). Sin embargo, el problema con este tipo de definición, es que comúnmente se piensa que para estar en homeostasis, hay un solo punto de ajuste exacto, lo que presupone que si algún parámetro se desvía de este valor exacto, se debe a que hay algún problema con los mecanismos internos.

Por esta razón fue que en 1988 Sterling y Eyer introdujeron el concepto de alostasis, que enfatizan dos puntos clave sobre los procesos de regulación: 1) los parámetros varían, y 2) la variación anticipa la demanda (2). Actualmente, una de las definiciones más aceptadas es la propuesta por McEwen y Wingfield en 2003; ellos definen al estrés como: los eventos que amenazan a un individuo y que provocan diversas respuestas fisiológicas y conductuales como parte de la alostasis, además de las impuestas por el ciclo de vida normal (2).

Por todo lo anterior, el estrés se ha considerado como una fuerza selectiva importante, que modula las respuestas adaptativas de las poblaciones naturales en un contexto ecológico y evolutivo (1,3–5). En condiciones naturales, los organismos son continuamente desafiados por diferentes factores de estrés; y la respuesta al estrés orquesta un conjunto de estrategias conductuales y fisiológicas que permiten a los animales enfrentar dichos retos estresantes (1,6,7). Sin embargo, si la respuesta al estrés es inadecuada o el estímulo se prolonga por un

periodo muy largo de tiempo (estrés crónico), esté podría tener consecuencias perjudiciales para la salud y la adecuación biológica del individuo (8,9). Por lo que, entender las capacidades fisiológicas de los organismos ante diferentes factores de estrés derivados del ambiente, se ha convertido en foco de interés para ecólogos y fisiólogos.

El estrés ambiental se entiende como la respuesta a las particularidades físicas del medio ambiente. El estrés que resulta de los cambios en los factores abióticos, como la temperatura, los factores climáticos y los componentes químicos, ya sean naturales o provocados por el hombre, se considera uno de los agentes estresores más importantes en la actualidad (10,11). Además, el estrés ambiental es un factor selectivo importante, que moldea la variación intraespecífica en las historias de vida de los organismos (10). Ya que la respuesta inicial al estrés con respecto a cambios en el medio ambiente depende predominantemente del nivel de variación genética permanente (standing genetic variation) de la población, los organismos con mayor diversidad alélica presentan una mayor capacidad de adaptación (12). Por lo que en este trabajo se propone que los organismos adaptados a medios ambientes extremos o altamente fluctuantes, tendrán respuestas más eficientes ante estímulos de estrés posteriores.

Existen varios aspectos de los mecanismos fisiológicos de respuesta al estrés que son potencialmente cuantificables y que han sido usados extensamente en el área para evaluar este tipo de respuestas. Es por eso que en esta tes

is está enfocada en cuantificar tres parámetros fisiológicos diferentes para evaluar la respuesta al estrés y el bienestar en general de los organismos: Los cambios en el estado redox, los niveles de glucocorticoides y la respuesta inmune.

Especies reactivas de oxígeno y estado redox

Los organismos aerobios usan al oxígeno como aceptor final de electrones durante la producción de energía. Este puede causar la producción de derivados del oxígeno, como los

radicales libres (radical hidroxilo: -OH, radical superóxido: O_2•-) y las especies reactivas no radicales (peróxido de hidrógeno: H_2O_2), llamadas en conjunto especies reactivas de oxígeno (ERO). Las ERO son moléculas parcialmente reducidas, por lo que son capaces de "robar" electrones de otras moléculas, lo que produce daño celular, como la peroxidación de lípidos, la oxidación de proteínas y el daño a los ácidos nucleicos (13–19). En condiciones de equilibrio, las ERO son moléculas esenciales para la señalización y regulación celular de diferentes funciones, tales como el crecimiento, la diferenciación, la proliferación y la apoptosis (20–22). La mayoría de las ERO en organismos vivos se producen en la cadena de transporte de electrones durante la respiración mitocondrial (17,23). Sin embargo, bajo circunstancias estresantes se incrementa su producción, por lo que el equilibrio entre la generación de ERO y su eliminación se altera. Lo que provoca un desequilibrio en el estado redox, por lo que el organismo entra en un estado estacionario llamado "*estrés oxidativo*" (24).

Los organismos han desarrollado mecanismos para mitigar los efectos tóxicos de las ERO mediante la regulación positiva de las enzimas antioxidantes (23), que juegan un papel importante en la eliminación de dichas moléculas. Por lo que el evaluar los niveles de las enzimas antioxidantes con respecto a la producción de ERO o del daño celular producido por estas, es una forma de evaluar la respuesta al estrés en organismos de forma *a priori* o a *posteriori,* en especies de vida silvestre en general (9,25–27). Estas características importantes hacen que los marcadores del estado redox sean herramientas informativas.

Glucocorticoides y respuesta inmune.

Por otro lado el eje hipotálamo-pituitario-adrenocortical (HPA) es una vía fisiológica ampliamente conservada en vertebrados, y desempeña un papel crucial como primera línea de defensa ante factores de estrés (28,29). La cascada del eje HPA produce y libera la hormona liberadora de corticotropina (CRH), que posteriormente estimula la síntesis y liberación de

adrenocortiocotropina (ATCH) en la pituitaria anterior, que a su vez estimula la síntesis y liberación de glucocorticoides (cortisol o corticosterona) en la corteza suprarrenal [29,30]. Los glucocorticoides (GCs) o también conocidas como las hormonas del estrés, son biomarcadores clave para evaluar la respuesta fisiológica de los animales a las perturbaciones ambientales, y se han utilizado como indicadores de la salud de la población animal en estudios de conservación (28,30–33). La principal acción de los GCs inducidos por el estrés es reorientar la asignación de energía hacia la supervivencia inmediata, a través de sus efectos estimulantes sobre el metabolismo, ya que los GCs pueden estimular la gluconeogénesis, la glicogénesis, la proteólisis y la lipolisis (34–36). Finalmente, otro biomarcador importante es la respuesta inmune, ya que se sabe que se afectad por eventos estresantes, como las fluctuaciones en las condiciones ambientales (27,37,38), los cambios de temperatura (39–44) y la falta de alimento (33–36). Además, se ha reportado que los GCs pueden tener un efecto inhibidor o potenciador sobre la respuesta inmune dependiendo el tiempo de exposición al estrés y el parámetro inmunológico analizado (7,49–51), por lo que relacionar la respuesta inmune con los niveles de GC permite tener un panorama más completo.

Por lo tanto, usar la información sobre las hormonas de estrés, la inmunocompetencia y el estado redox en conjunto, es importante para una mejor caracterización de la respuesta al estrés de las poblaciones de animales ante las perturbaciones ambientales. Así mismo, esto es relevante para evaluar la importancia relativa de cada parámetro fisiológico en la predicción de dichas respuestas en condiciones ambientales fluctuantes.

Este trabajo está enfocado a estudiar el papel del estrés ambiental en relación a los aspectos fisiológicos basales con los que cuentan los animales que viven en ambientes potencialmente estresantes, así como su respuesta fisiológicas ante estímulos de estrés, mediante la medición de glucocorticoides, enzimas antioxidantes, e inmunocompetencia. Además, se determino la interacción de dichos parámetros fisiológicos. Uno de los temas centrales de interés en este

trabajo fue la estacionalidad. Existen múltiples estudios que se han enfocado en determinar cambios estacionales en los niveles basales de GCs (52–57), antioxidantes (58–63) y respuesta inmune (55,64–67). Sin embargo, los diferentes requerimientos energéticos que tienen los organismos a lo largo del año por su historia de vida (e.g. actividad reproductiva, uso del torpor etc.), además de las diferentes condiciones ambientales (temperatura alta o baja, tormentas, falta de alimento, etc.) a las que pueden estar expuestos, puede influenciar la respuesta fisiológica al estrés de los organismos.

Objetivos de los capítulos I, II y III.

Los primeros dos capítulos de la tesis se enfocan en estudiar el efecto de la estacionalidad en los procesos fisiológicos basales y post-estrés en el murciélago *Myotis vivesi*. Esta especie es endémica de islas desérticas en el golfo de Baja California, donde la temperatura ambiental oscila entre 50°C en verano y 5°C en invierno, y hay presencia de fuertes vientos y tormentas en la época invernal que limita el forrajeo. Tal variación a lo largo del año, añadido al potencial estrés ambiental que estas condiciones pudieran tener sobre la población, nos da un *continuum* de adecuación no lineal. Considerando que la temperatura y el viento varían drásticamente a lo largo del año; los murciélagos deberían de estar en su adecuación máxima a temperaturas y vientos de intensidad intermedia. Mientras que en los extremos de calor y frío la adecuación biológica sería baja y por lo tanto la exposición al estrés mayor; además durante dichos extremos hay una incidencia con actividades energéticas de rasgos reproductivos por lo que esperaría que la respuesta al estrés disminuyera.

El capitulo I de la tesis consiste del artículo requisito del programa de posgrado en ciencias biológicas, titulado **“Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat *Myotis vivesi*”**. El objetivo de este trabajo fue determinar el estado redox y la variación basal inmune durante el verano, otoño e invierno en

Myotis vivesi y B) determinar el efecto que tiene un estímulo de estrés agudo sobre la inmunocompetencia y el estado redox en cada estación.

El capítulo II se tituló: **"Glucocorticoids seasonal variation and its relation to immunocompetence and oxidative stress in an endemic insular bat".** En este estudio se describió el perfil hormonal de los metabolitos del cortisol de *M. vivesi* y su respuesta a un estrés agudo a lo largo del año. Además, se determinó la relación de los GCs en la respuesta antioxidante e inmunológica después de un estímulo agudo (endógeno, inducido por la restricción mecánica del movimiento por 24 h), y después de un estímulo crónico (exógeno, inducido por la inyección de ACTH y su monitoreo hasta por 48 h).

En la tercera parte de la tesis (capítulo III), se estudiaron los cambios en el estado redox de los organismos que utilizan la hibernación como parte de sus historias de vida. La incapacidad de las especies que no hacen uso del torpor de poder sobrevivir a condiciones como hipotermia prolongada y/o supresión del metabolismo sugiere que aquellos que si están adaptados para la hibernación deben enfrentarse con riesgos fisiológicos significativos para asegurar la sobrevivencia durante y después de la temporada invernal, ya que al entrar en torpor corren el riesgo de sufrir daños fisiológicos. Durante los despertares periódicos a la eutermia, la frecuencia cardíaca, la ventilación y el metabolismo aumentan antes de que la temperatura corporal (T_c) haya sido completamente restablecida, lo que potencialmente aumenta el riesgo de daño oxidativo. El capitulo III de la tesis se titula **"Redox state changes in several tissues from *Myotis velifer* after hibernation interruption"** y su objetivo fue determinar cambios en el estado redox y daño oxidativo al interrumpir el ciclo de hibernación natural en *Myotis velifer*, un murciélago heterotérmico que hiberna de septiembre a marzo. Los murciélagos fueron capturados directamente de su hibernáculo en Tecomalucan, Tlaxcala; al final de la temporada de hibernación. Se determinaron las enzimas antioxidantes CAT, SOD y GPx, y el daño tisular determinando el contenido de carbonilos en sangre, cerebro, corazón, pulmones,

hígado y TAB en 5 murciélagos en hibernación (temperatura corporal = 11ºC) y en 5 murciélagos después de salir del estado de torpor (temperatura corporal = 30ºC) para evaluar si ocurrió estrés celular al momento de interrumpir el ciclo de torpor.

A continuación se presentan los tres artículos referentes a los capítulos mencionados arriba y una conclusión general.

CAPITULO I:

ARTÍCULO REQUISITO

Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat *Myotis vivesi*

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PLOS ONE

RESEARCH ARTICLE

Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat *Myotis vivesi*

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Abstract

Little is known of how the stress response varies when animals confront seasonal life-history processes. Antioxidant defenses and damage caused by oxidative stress and their link with immunocompetence are powerful biomarkers to assess animal´s physiological stress response. The aim of this study was A) to determine redox state and variation in basal (pre-acute stress) immune function during summer, autumn and winter (spring was not assessed due to restrictions in collecting permit) in the fish-eating Myotis (*Myotis vivesi*; Chiroptera), and B) to determine the effect of acute stress on immunocompetence and redox state during each season. Acute stress was stimulated by restricting animal movement for 6 and 12 h. The magnitude of the cellular immune response was higher during winter whilst that of the humoral response was at its highest during summer. Humoral response increased after 6 h of movement restriction stress and returned to baseline levels after 12 h. Basal redox state was maintained throughout the year, with no significant changes in protein damage, and antioxidant activity was modulated mainly in relation to variation to environment cues, increasing during high temperatures and decreasing during windy nights. Antioxidant activity increased after the 6 h of stressful stimuli especially during summer and autumn, and to a lesser extent in early winter, but redox state did not vary. However, protein damage increased after 12 h of stress during summer. Prolonged stress when the bat is engaged in activities of high energy demand overcame its capacity to maintain homeostasis resulting in oxidative damage.

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Competing interests: The authors have the following interests: Alejandra Ibáñez-Contreras is employed by Unidad de Experimentación Animal, Biología Integral para Vertebrados (BIOINVERT®). There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

Animals confront stressful situations throughout their lives, whether due to changes in environmental conditions or to predation and competition [1,2]]. Environmental stress has a significant impact on evolutionary and ecological processes that affect and shape population genetic structure and evolution [3]. The former situations are potentially amplified by human activities due to habitat loss or alteration [4], causing rapid and often stressful and deleterious changes. Therefore, assessing animal responses to stress has a practical relevance for determining the impact of human activities (e.g. ecotourism, recreational fishing, vehicle traffic), or conservation interventions (e.g. animal trapping, restraint and handling, trans-location and radio-collaring, and species management programs) on animal well-being [5,6].

Different biomarkers to assess animal´s health in the wild have been reported; however, hormones dynamics, metabolic rate, and the magnitude and intensity of the immune response have become the gold standard of field ecologists [7–9]. Recently, determining redox state changes has also become an important tool in ecological studies [10]. Animals use oxygen to produce energy, which ultimately leads to production of free radicals along with non-radical reactive oxygen species, collectively called reactive oxygen species (ROS). These molecules induce cellular damage, such as lipid peroxidation, protein oxidation, and nucleic acid damage [11–16]. Enzymatic antioxidants, such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and, along with the three-peptide Glutathione (GSH), are the main line of defense promoting ROS removal [17]. Nevertheless, certain cellular functions, such as redox signaling [18], rely upon ROS; hence, antioxidants maintain an optimum oxidant level rather than eliminate them entirely [19].

The importance of the immune response as a powerful biomarker to asses stress relies on the fact that changes in the endocrine system after stress episodes have paramount consequences on animal immune function. Stressful events, such as environmental condition fluctuations [20–23], cold temperatures [24–29] and lack of food [30–32] also affect an animals immunity, making this marker very useful to assess stress [33]. Furthermore, modifications in redox state have an important role during an immune response. Animal metabolic rate is usually elevated during the immune response [34], due in part to higher mitochondrial activity and consequently correlated with increased ROS production [35]. Additionally, leukocytes involved in the immune response, in particular neutrophils, produce ROS to eliminate pathogens [36] and to enhance T-lymphocytes activation [36,37]. Thus, assessing both redox state and immunocompetence provides a more integrated outlook of animal health state. Although several studies have measured antioxidant response [38–44] and immune variation [45–49] in wild animals during different seasons, they have used baselines measurements, and research on the effect of acute stress in these physiological parameters throughout the year is mostly inexistent. There are some experiments where redox changes [50] and cellular immunity [51–53] after acute stress have been determined under laboratory experimental conditions and some others where the effect on humoral immune response has been reported in the wild [9,54,55]. However, the results of these studies are contradictory. These contradictions probably arise from the differences in sample collecting conditions and the season when the samples were collected. Animals are engaged in different activities through the year with contrasting energy demands (i.e reproduction in spring/summer, or torpor during winter, etc.) and are exposed to different environment conditions (i.e high or low temperature, storms, lack of food, etc.) that might affect their redox state and immunocompetence. Accordingly, in this study we tested if the response to acute stress is related to the environment conditions when the stressful stimuli is presented and consequently to the season when it was measured, taking into account individual´s body condition, sex and reproductive state. We conducted this study with the Myotis fishing bat *Myotis vivesi*, Menegaux, 1901, endemic to the Gulf

of California, Mexico. Ambient temperature (T_a) in the islands where this bat roosts range from 45ºC in summer to 5ºC during winter [56]. During daytime, bats roost under rocks, which barely provide them minimal isolation from T_a [57]. Lactation in this bat occurs during summer, and during winter these bats use torpor [57]. Torpor increases the risk of oxidative stress damage during the rewarming periods, so that the animals adapted to this process must have strong antioxidant mechanisms [58]. Thus, *M. vivesi* represents an ideal natural model to test the following objectives: A) to determine seasonal variations in redox state and basal (pre-stress stimulus) immune function, and B) to determine the effect of acute stress on immunocompetence and redox state in different seasons. Redox state was determined by measuring systemic antioxidant enzymatic activity (AEA) and cellular damage (protein carbonyls). Bat immunocompetence was determined by measuring the cellular immune response (Phytohaemagglutinin test- PHA) and humoral immune response (bactericidal activity of plasma- BA). Bats were subjected to 6 and 12 h of movement restriction as a source of stress, and the change in their physiological parameters was monitored after each stress period.

Our results showed that the basal immune response was an important physiological response that changed through the year, with a trade off in functionality of immune components. The cellular immune response was higher during winter whilst the humoral response was higher in summer. Humoral response increased after 6 h of movement restriction stress and returned to baseline levels after 12 h. Basal redox state was maintained through-out the year, with no significant changes in protein damage, and the antioxidant activity increased during high temperatures and decreased during windy nights. Bats responded to the 6 h acute stress by enhancing their antioxidant activity especially during summer and autumn, and to a lesser extent in early winter, with no changes in redox state. However, some protein damage began to accumulate after 12 h of movement restriction stress during summer, when more than one energy demanding process concur, (i.e. humoral immune response, AEA response, thermoregulatory demands due to high temperatures, and lactation). All the above suggests that stress overload during highly energetically-demanding periods might force the system and produce cellular damage.

Materials and methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The reagents obtained from other sources are detailed throughout the text.

Study site

This study was conducted in Partida Norte Island (28º52'30"N, 113º21'7"W), a 1.4-km^2 island located in the midriff region of the Gulf of California, Mexico [59]. This island is home to the largest known colony of *Myotis vivesi* (~8,000 adults)[60]. Fieldwork was carried out for five days during each visit for two consecutive periods. The first period was during July, October and December 2014, and February 2015. The second period was during June and October 2015, and February 2016. Those months were selected in order to sample animals in summer when lactation occurs, in winter when bats use torpor, and in autumn, a transition period between these two activities. We did not sample in spring when the colony is mostly composed of pregnant individuals [61] due to restrictions in our research permit.

Sample collection

Only adult bats were captured between 6:00–7:00 am directly from their roost site under rockslides. They were kept individually in small cotton bags, where the acute stress stimuli

consisted of their immobilization within the bag. It has been reported that capture, handling and immobilization induces stress as shown by increase in glucocorticoid levels [62–65]. We also measured cortisol levels in fecal samples collected $\leq$ 6 h and $\geq$ 7 h after the animal was captured. Based on observations of gut transit time in our focal species, we considered that feces collected in the first period were formed before capture; thus, the effect of capture stress on cortisol levels is most likely reflected on feces collected in the second period. Accordingly, feces collected in the first period had significantly lower cortisol levels than those collected in the second period (data not shown). A basal blood sample (150 μl) was obtained from all bats after capture by bleeding from the right forearm vein. Afterwards, bats were randomly selected to form two groups: animals stressed for 6 h and animals stressed for 12 h. The second blood sample (150 μl) was drawn from the left forearm vein 6 or 12 h after capture, depending on the animal group. Blood was drawn by punctuation with a hypodermic needle (27Gx 13mm BD), collected in heparinized capillary tubes and placed in a 1.5 mL eppendorf tube. After 24 h, bats were returned to the place where they were captured. All samples were kept on ice during collection and handling, and plasma and erythrocytes were separated after centrifugation at 6000 rpm (Digital ZipSpin Centrifuge LWScientific), stored in 0.2 mL tubes and frozen in liquid nitrogen. Afterwards, samples were transferred from liquid nitrogen to dry ice and shipped to the laboratory where they were stored at -80°C until their analysis.

We measured body mass with a portable XSXScale ES200G x 0.01G (± 0.01g) and forearm length with Vernier calipers (Mitutuyo CD-6″CSX; ± 0.01mm). Sex was recorded for all bats captured. Only adult animals were used and they were differentiated from young individuals by examining their ossification index. Accordingly, we exposed the wing of each individual to transillumination using a headlight; in young individuals, the cartilaginous zone of the long phalanges is visible because less mineralized tissue allows more light to pass through and thus appears lighter than bone. When bats reach adulthood, epiphyseal plates eventually close and are no longer visible to the unaided eye. Fur color was also an indicator of age; adults have brown-gold fur while young have gray fur [66]. A total of 116 bats were captured during the first period of sampling (P1) (summer: 32 females, 6 males; autumn: 11 females, 5 males; early winter: 24 females, 11 males; late winter: 13 females, 14 males) and 117 during the second period of sampling (P2) (summer: 34 females, 6 males; autumn: 24 females, 13 males; late winter: 26 females, 14 males).

This study was carried out in strict accordance with the recommendations and permits approved by Mexican Government (Secretaría de Gobernación #013/13) and from Dirección General de Vida Silvestre (#01947/13), Mexico. All sampling procedures and experimental manipulations were performed according to the Principles of the Mexican Official Ethics Standard 062-ZOO-1999 and were approved as part of obtaining this permit. No other approval was required to conduct the study, as there is no IACUC/animal ethics board at our institution.

Weather data

Data for ambient temperature (T_a) and wind speed (W) were obtained from the nearest meteorological station in Bahía de los Ángeles, Baja California, Mexico. Data was registered every 10 sec. Considering that the rock roost does not completely insulate bats from T_a [57], we used the total daily data recorded to obtain the daily median ambient temperature ($d\bar{T}_a$) of each sample day. Considering that bats are mostly affected by wind during their foraging hours, we used the data recorded between 19:00 and 23:59 hours to obtain the median night wind speed ($\hat{W}_n$) of the sampled days.

Body condition determination

Ecologists have measured body condition as a non-destructive method to estimate nutritional state and provide a snapshot of an animal's physiological state [67]. Here, body condition was determined with two methods. First, we used the Scaled Mass Index (SMI) [68] which relies on measures of body mass and linear measures of body size to calculate a condition index with the following formula:

$$\hat{M}i = Mi\,[L_0/L_i]^{bSMA}$$

where Mi is the weight (g), L_i is the forearm length (mm), L_0 is the forearm arithmetic mean, bSMA is the scaled exponent estimated using online software [69], and ^Mi is the predicted body mass for an individual when the lineal body measurement is standardized to L_0. The second method used to calculate body condition was the hematocrit percentage (%H). Hematocrit is defined as the percentage of the total blood volume occupied by erythrocytes, which depends on the variation in plasma volume, the rate of erythrocyte production and destruction, dehydration, toxins, and direct blood loss, and it may hence be used as an index of the 'health' of the oxygen transport system. It was calculated by dividing the total blood draw volume by the erythrocyte volume of each blood sample. This was done with the basal blood samples and the post stress blood samples (6 and 12 h).

Phytohemagglutinin challenge

The delayed cutaneous hypersensitivity response was quantified as an indicator of cellular immunity responsiveness [70–72]. This response was assessed by injecting 50 μL of a phytohemagglutinin (PHA) solution (3 mg PHA/mL of phosphate buffered saline-PBS) on the right foot, and 50 μL of PBS on the left foot. PHA influences a variety of cell types and, therefore the response to PHA injection is complex, but can serve as an index for heightened immune cell activity [73,74]. Thickness of the foot was measured before injection and 6, 12 and 24 h afterwards using digital calipers (Mitutuyo CD-6″CSX (± 0.01mm). Cellular immune response was calculated as the change in thickness of the PHA injected foot minus the change in the control foot [75,76]. Larger localized swelling indicates increased immune activity. Measurements were made in triplicate and the mean was used for analyses.

Bactericidal activity (BA)

The antimicrobial capacity of plasma was assessed with the Liebl and Martin Ii protocol [77] as a measurement of serological components [78] (non-specific antibodies [79], complement cascade [80,81], and lysozyme activity [82]). Before assay, the bacteria *Escherichia coli* (ATCC #8739) were reconstituted according to manufacturer instructions. Stock solution of the bacteria was diluted to 1 x 10^5 microbes mL^{-1}. Plasma was diluted in sterile PBS 1:23, 25 μL of working solutions was added, and samples were incubated for 30 min at 37°C. After the first incubation, 500 μL of Soy Broth (TSB) was added. Samples were incubated a second time at 37°C for 12 h. After the second incubation the samples were analyzed spectrophotometrically (Beckman DU-650) at 340 nm. The portion of killed bacteria was calculated as 1- (Sample Abs_{340} / control Abs_{340}). All samples were analyzed in duplicate.

Protein extraction

Erythrocytes samples were first washed twice with 0.9% NaCl by centrifugation at 3000 rpm for 10 min. Protein was extracted afterward by adding lysis buffer (100 μL DTT 1M, 100 μL Phenylmethylsulfonyl flouride (PMSF) 0.1M, 1 cOmplete™ tablet, 10 mL T-PER) and

centrifugated at 13500 rpm at 4°C for 15 min. Protein samples were separated in four aliquots to prevent frizzing and thawing. Before each assay, total protein concentration in each aliquot was determined spectrophotometrically at 595 nm using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) [83].

Antioxidant enzyme activity

Antioxidant enzyme activity (AEA) was analyzed spectrophotometrically (Thermo Scientific™ GENESYS 10S UV-Vis; Madison, WI USA) in erythrocyte protein samples as described elsewhere [84]. Briefly, SOD activity was determined through the xantine/xantine oxidase system, based on protocols by Paoletti *et al* [85]. The superoxide anion formed through this system reacts with the nitro blue tetrazolium (NBT) and generates a formazan salt that was measured spectrophotometrically at 560 nm every 30 sec for 5 min. One unit of enzymatic activity in this assay is considered as the amount of SOD needed to inhibit 50% of the superoxide reaction with NBT. CAT activity was quantified using the protocol established by Abei [86], which evaluates the decline in absorbance at 240 nm, as H_2O_2 is catalyzed to H_2O and O_2, every 15 sec for 3 min. One unit of catalase activity (UCAT) was considered as the amount of enzyme necessary to catalyze 1 μmol of H_2O_2 per minute. GPx activity was analyzed at 340 nm through a protocol described by Ahmad *et al* [87]. One unit of GPx activity indicates how much enzyme is required to neutralize H_2O_2 using NADPH [88].

Protein oxidative damage

Carbonyl concentration was determined in order to assess protein oxidative damage by using the DNPH alkaline method [89] and adjusting the optimal volumes for its use in 96 wells plates. 20 μL of DNPH (10 mM in 0.5 M H_3PO_4) were added to 20 μL of sample protein. Samples were incubated for 10 min in the dark with constant agitation. Afterwards, 10 μL of NaOH (6M) were added and incubated in the dark for 10 min at room temperature. Absorbance was determined at 450 nm against a blank where the protein solution was substituted by an equal volume of buffer solution. Carbonyl content was calculated as (Abs_{450}/E)/total protein content of sample, where E = extinction factor of 46.1.

Statistical analysis

All data were tested for normality for univariate analysis, with the D'Agostino & Pearson omnibus normality test and for homoscedasticity with Levene tests. When data met normality and homoscedasticity assumptions, an analysis of variance (ANOVA) was performed followed by a Tukey´s post hoc test when needed. When normality was met but homoscedasticity was not, an ANOVA with Welch correction and a post hoc Games-Howell test were conducted. When neither criterion was met, then we used a Kruskall-Wallis test followed by Dunn's post hoc test if needed. All data were tested for differences between sexes with either a t-test or Mann-Whitney U test. Significant differences between sexes were found only for the PHA index on P1. Thus, an ANCOVA (Sex × Season) with scaled mass index as a covariate was performed to compare the PHA index. The stress stimuli effect was analyzed with a t-test. Principal component analysis (PCA) was conducted using the software PAST version 2.17c. Only data from individuals that had all variables measured were included in the analysis. All 12 variables were included (BA, SOD, CAT, GPx, PHA, SMI, %H, carbonyls, $d\mp_{a,}$ $\hat{W}_{n,}$ sex and reproductive state: rep) for baseline analyses. We used the mean value for the ambient variables, from the day previous to individual collection. For post stress analyses of 6 h and 12 h, we included (BA6/12, SOD6/12, CAT6/12, GPx6/12, %H6/12, Carbonyls6/12, sex, and rep. All data included in the PCA´s had a normal multivariate distribution (Mardia test).

Principal component analysis baseline analyses (D-PCA) were computed in a correlation matrix to determine which variables had more weight on the model and how the variables interacted with each other during periods 1 and 2 separately. Variables with coefficient values < 0.5 were considered not to significantly contribute to the model and were removed from the analysis. Afterwards, a between-group analysis (BG-PCA) was computed in the correlation matrix in order to highlight the difference between groups (seasons) by measuring the contribution (in terms of variance) of the variables in the differentiation of individuals between groups.

Results

Weather conditions

For period 1 (P1), daily mean ambient temperature ($d\bar{T}_a$) was different among seasons sampled ($H_3 = 1582$, $P < 0.0001$). Summer had the highest $d\bar{T}_a$ (median, minimum-maximum: 26.4ºC, 22.4–39.2ºC) compared to autumn (25.9ºC, 22.3–30.2ºC; $P = 0.0075$), early winter (20.0º C, 16.3–24.4ºC; $P < 0.0001$) and late winter (21.2ºC, 16.7–25.4ºC; $P < 0.0001$). Autumn $d\bar{T}_a$ was higher than in early winter ($P < 0.0001$) and late winter ($P < 0.0001$), whereas $d\bar{T}_a$ from late winter was higher than $d\bar{T}_a$ from early winter ($P = 0.003$). For period 2 (P2), $d\bar{T}_a$ was also different among seasons ($H_2 = 1097$, $P < 0.0001$). Summer (28.6ºC, 21.0–41.2ºC) was hotter than autumn (26.0ºC, 22.3–30.2ºC; $P < 0.0001$) and late winter (20.5ºC, 15.7–26.1ºC; $P < 0.0001$), and autumn was warmer than late winter ($P < 0.0001$) (Fig 1A). Both periods showed the same pattern, with the highest temperature in summer and the lowest in winter, but summer $d\bar{T}_a$ was significantly higher in P1 than P2 ($H_6 = 2453$, $P < 0.0001$).

Median night wind speed ($\hat{W}_n$) was significantly different ($H_3 = 100.6$, $P < 0.0001$) among P1 seasons: summer had the lowest $\hat{W}_n$ (12.4 km/h, 2.9–31.7 km/h) in comparison to autumn (25.1 km/h, 3.6–50.5 km/h; $P < 0.0001$), early winter (19.5 km/h, 3.3–27.4 km/h; $P < 0.0001$), and late winter (25.2 km/h, 7.2–35.1 km/h). $\hat{W}_n$ was higher at the end of winter than at the beginning ($P = 0.0004$). For period 2, there were also significant differences among seasons ($H_2 = 31.8$, $P < 0.0001$). $\hat{W}_n$ was higher during autumn (25.9 km/h, 5.3–52.8 km/h; $P < 0.0001$) and late winter (20.2 km/h, 6.3–28.5 km/h; $P = 0.001$) than during summer (16.2 km/h, 3.8–

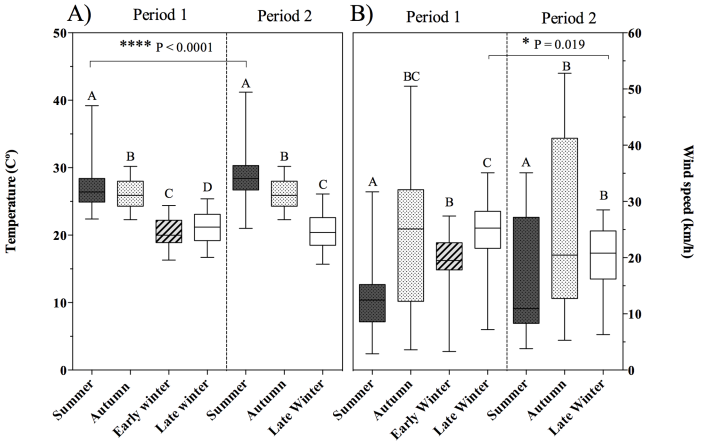

Fig 1. Weather conditions by season on both periods (period 1 and period 2) of sample collection. A) Temperature (ºC) (median ± interquartile range); B) Wind speed (km/h) (median ± interquartile range). Different letters indicate significant differences among seasons within a period and asterisks indicate significant differences between the same seasons across different periods, as determined by Kruskal-Wallis analysis of variance.

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35.0 km/h) (Fig 1B). Both periods showed the same kind of outline ($H_6 = 123.9$, $P < 0.0001$), but $\dot{W}_n$ in late winter was significantly lower in P2 than in P1 ($P < 0.019$) (Fig 1B).

Body condition

Hematocrit varied across seasons during P1 ($F_{3, 116} = 27.7$, $P < 0.0001$), with higher values during summer and autumn than in early winter ($P < 0.0001$) and late winter ($P < 0.0001$). Hematocrit also varied in P2 ($F_{2, 108} = 10.6$, $P < 0.0001$) with lower hematocrit in late winter than in summer ($P < 0.011$) and autumn ($P < 0.0001$). There were significant differences in hematocrit between periods ($F_{6, 222} = 15.9$, $P < 0.0001$) but only for summer with a higher value in P1 ($P = 0.004$) (Fig 2A).

The scaled mass index (SMI) varied significantly among seasons during P1 ($F_{3, 104} = 3.9$, $P = 0.010$): it was significantly higher in autumn that in early winter ($P = 0.045$) and late winter ($P = 0.007$), but not significantly different from summer ($P = 0.261$). No significant differences

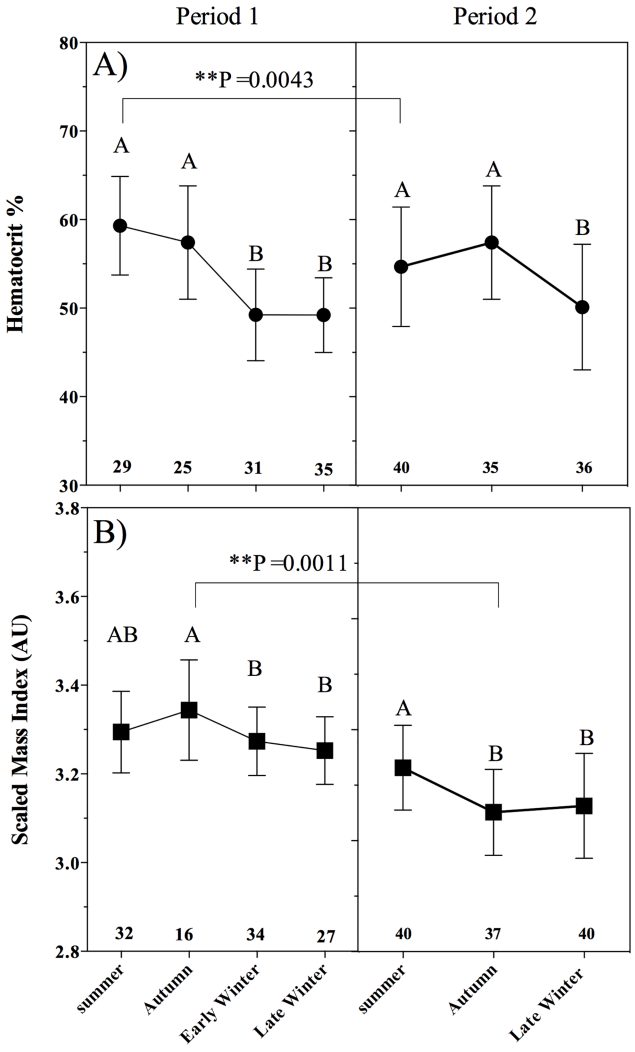

Fig 2. Bats body condition by season on both periods (period 1 and period 2) of sample collection. A) Hematocrit percentage (mean ± SD); B) Scaled mass index (mean ± SD). Different letters indicate significant differences between seasons within a period and asterisks indicate significant differences between the same seasons of different periods as determined by ANOVA. Numbers in bold indicate sample sizes.

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were found in SMI between summer and early winter (P = 0.774) or late winter (P = 0.272). There were significant differences among seasons in P2 ($F_{2, 114}$ = 10.6, P < 0.0001) but in a contrasting pattern; the SMI was lower in autumn than in summer (P = 0.0001) and late winter (P = 0.001). The SMI varied significantly between periods ($F_{6, 218}$ = 5.47, P < 0.0001) but only in autumn during which the index was higher in P1 (P = 0.007) (Fig 2B).

Seasonal enzyme antioxidant activity and protein damage

Basal SOD activity varied significantly across seasons during P1 ($F_{3, 34.9}$ = 51.2, P < 0.0001), with higher values in autumn than in early winter (P < 0.0001), late winter (P = 0.011) and summer (P = 0.001); in turn, basal activity during early winter was higher than in summer (P < 0.0001) and late winter (P < 0.0001), while activity during summer was higher than in late winter (P < 0.0001) (Fig 3A). There were significant differences among seasons in P2 ($F_{2, 58.4}$ = 3.7, P = 0.030), with higher values in autumn than in late winter (P = 0.028) and a trend towards a higher value than in summer (P = 0.061), and no significant difference between summer and late winter (P = 0.716) (Fig 3B). Basal SOD activity during summer (P < 0.0001), and autumn (P < 0.0001), was higher in P1 than in P2 ($F_{6, 65.7}$ = 33.4, P < 0.0001), and no other significant difference was found between periods for other seasons (Fig 3A and 3B).

CAT basal activity varied significantly among seasons in P1 ($F_{3, 29.2}$ = 7.4, P = 0.001) with higher values in autumn than summer (P = 0.028), early winter (P = 0.11), and late winter (P = 0.004). No difference in CAT activity was found between summer and early winter (P = 0.97) and late winter (P = 0.48) (Fig 3A). CAT activity varied significantly among seasons in P2 ($F_{2, 66.4}$ = 124.95, P < 0.0001) but with a contrasting pattern: activity was higher in summer than autumn: (P = 0.001) and late winter (P < 0.0001), and higher in autumn than late winter (P < 0.0001) (Fig 3B). Differences between periods ($F_{1, 61.8}$ = 52.1, P < 0.0001) in CAT baseline levels were significant only in autumn when P2 had a higher value (P < 0.0001) (Fig 3A and 3B).

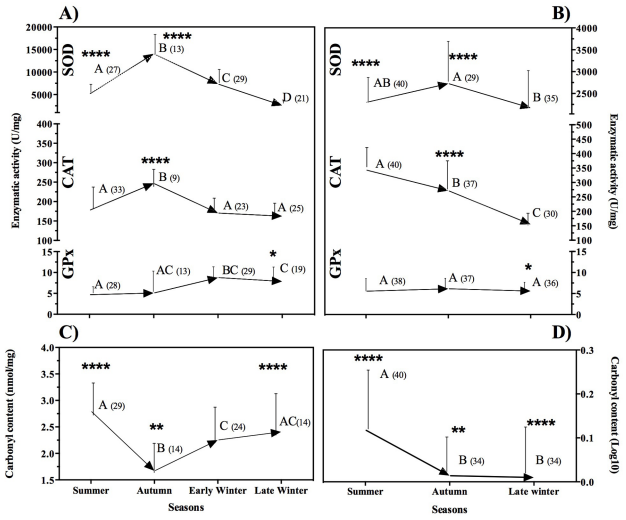

Fig 3. Oxidative stress on bats by season for both periods (period 1 and period 2) of sample collection. A) P1 antioxidant enzymatic activity in erythrocytes (mean ± SD). B) P2 antioxidant enzymatic activity in erythrocytes (mean ± SD). C) Protein damage measured by carbonyl quantification in erythrocytes (mean ± SD) on P1. D) Protein damage measured by carbonyl quantification in erythrocytes (mean ± SD) on P2. Different letters indicate significant differences among seasons of a period as revealed by ANOVA with Welch correction. Numbers in parenthesis indicate sample sizes.

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GPx basal activity varied significantly among seasons in P1 ($F_{3, 30.9} = 14.8$, $P < 0.0001$): activity was higher in early winter than in summer ($P < 0.001$) and autumn ($P = 0.001$), and not significant difference was found in GPx activity between early winter and late winter ($P = 0.10$) (Fig 3A). In contrast, no significant differences among seasons were found in P2 ($F_{2, 108} = 0.7$, $P = 0.49$) (Fig 3B). No significant differences were found between periods for any season ($F_{6, 64.2} = 7.6$, $P < 0.0001$; autumn P1vs P2 $P = 0.991$; summer $P = 0.703$; late winter $P = 0.915$) (Fig 3A and 3B).

Protein damage varied significantly among seasons in P1 ($F_{3,77} = 9.1$, $P < 0.0001$): the highest protein damage was found in summer and late winter, with no significant difference between these seasons ($P = 0.154$), but both were significantly higher than in autumn (summer vs. autumn: $P < 0.0001$; late winter vs. autumn: $P = 0.023$). The damage in early winter was significantly higher than in autumn ($P = 0.037$), and significantly lower than in summer ($P = 0.023$), but no different than in late winter ($P = 0.49$) (Fig 3C). Protein damage varied significantly among seasons in P2 ($F_{2, 66.38} = 6.4$, $P = 0.003$) with significantly higher values in summer than in autumn ($P = 0.003$) and late winter ($P = 0.009$), and no difference between autumn and late winter ($P = 0.999$) (Fig 3D). Protein damage differed significantly between periods ($H_{7, 182} = 119.1$, $P < 0.0001$) with higher values in P1 for all seasons ($P \leq 0.005$) (Fig 3C and 3D).

Seasonal PHA challenge response

The ANCOVA model for PHA index after 6 h of the injection was significant on P1 ($F_{8, 96} = 2.8$, $P = 0.009$). After correcting for individual body conditions, the PHA response was significantly greater in females than males ($F_{1, 96} = 8.3$, $P = 0.005$). The magnitude of the PHA response did not differ significantly among seasons ($F_{3, 96} = 1.5$, $P = 0.23$) but the interaction between sex and season was significant ($F_{3, 96} = 3.1$, $P = 0.029$): females exhibited a more robust swelling response in summer than in early winter ($P = 0.046$) (Fig 4A).

The ANCOVA model for PHA index after 12 hours of the injection on P1 was not significant ($F_{8, 96} = 0.68$, $P = 0.71$); neither sex ($F_{1, 94} = 2.5$, $P = 0.12$) (Fig 5A), nor season ($F_{3, 94} = 0.9$, $P = 0.46$) (Fig 5B) had a significant effect on PHA swelling response. The interaction between sex and seasons was also non-significant ($F_{3, 94} = 0.5$, $P = 0.66$).

The ANCOVA model for PHA after 24 hours was significant ($F_{8, 94} = 2.7$, $P = 0.01$). The effect of body condition was not significant ($F_{1, 94} = 3.9$, $P = 0.758$), and after correcting for body condition the PHA swelling response was not significantly different between sexes ($F_{1, 94} = 3.9$, $P = 0.051$) (Fig 6A), but it did differ significantly among seasons ($F_{3, 94} = 4.1$, $P = 0.009$) (Fig 6B). The magnitude of the swelling response in late winter was higher than in summer ($P = 0.007$), but was not significantly different than in autumn ($P = 0.14$) or early winter ($P = 1.0$). The swelling response in summer was not different than in autumn ($P = 0.14$) or early winter ($P = 1.0$).

The ANCOVA model for P2 after 6 h injection was also significant ($F_{6, 105} = 5.3$, $P < 0.0001$). After correcting for body conditions of individuals, the magnitude of the PHA response was not significantly different between sexes ($F_{1, 105} = 0.4$, $P = 0.553$) (Fig 4B), but was different among seasons ($F_{2, 105} = 11.9$, $P < 0.0001$) (Fig 4C). The interaction between sex and season was not significant ($F_{2, 105} = 0.8$, $P = 0.452$). The model for the PHA index after 12 hours was significant ($F_{6, 106} = 5.9$, $P < 0.001$), but after correcting for the effect of body condition, significant differences were found among seasons ($F_{2, 106} = 12.3$, $P < 0.001$) (Fig 5D) but not between sexes ($F_{1, 106} = 0.5$, $P = 0.83$) (Fig 5C). The magnitude of the swelling after 12 h of injection was greater in late winter than in autumn ($P < 0.0001$) and summer ($P = 0.001$). The interaction between sex and season was not significant ($F_{2, 106} = 0.7$, $P = 0.52$). The model

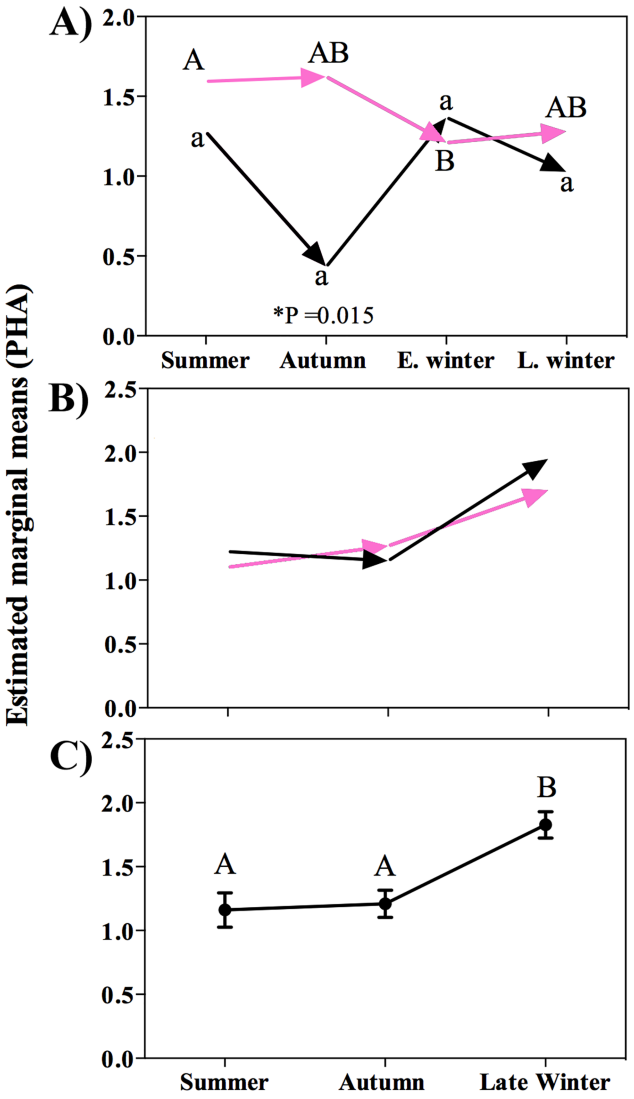

Fig 4. Seasonal swelling response after 6 hours of PHA post injection. A) P1, pink arrows represent seasonal variation in the swelling response in females; black arrows represent seasonal changes in the swelling response in males; different letters indicate significant differences among seasons within a period; asterisks indicate differences between periods for a given season. B) P2, pink arrows represent females and black arrows represent males; no significant differences were found. C) Combined data from P2 (estimated mean ± SE).

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describing the magnitude of the PHA response after 24 hours was significant ($F_{6, 97} = 9.9$, $P < 0.0001$). The effect of body condition was significant ($F_{1, 97} = 4.1$, $P = 0.047$).After correcting for the effect of body condition, we found no significant difference between sexes ($F_{1, 97} = 1.1$, $P = 0.307$) (Fig 6C), but swelling was significantly different among seasons ($F_{2, 97} = 24.1$, $P < 0.0001$) (Fig 6D). Similar to P1, swelling response was higher in late winter than summer ($P < 0.001$) and autumn ($P < 0.001$). The interaction between sex and season was not significant ($F_{2, 97} = 0.32$, $P = 0.715$).

Seasonal Bactericidal Activity (BA)

BA of plasma samples collected immediately after capture was different among seasons in P1 ($F_{3, 41.13} = 55.14$, $P < 0.0001$), with higher values in summer than autumn ($P < 0.0001$), early

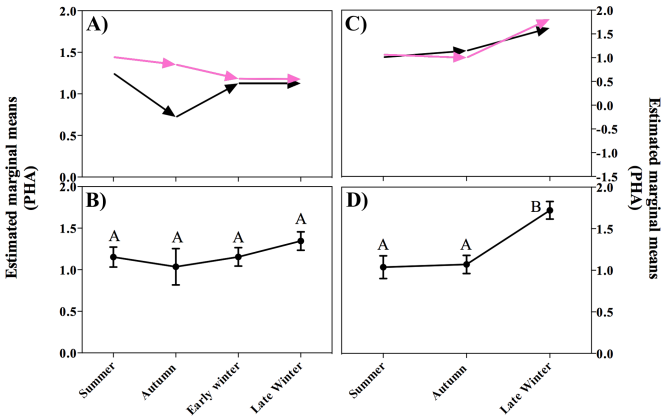

Fig 5. Seasonal swelling response after 12 hours of PHA injection. A) Data separated by sex from P1. B) Combined data from P1 (estimated marginal mean ± SE). C) Data separated by sex from P2. Pink arrows represent seasonal variation in the swelling response in females; black arrows represent seasonal changes in swelling response in males. D) Combined data from P2 (estimated marginal mean ± SE); different letters indicate significant differences among seasons.

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winter (P = 0.003), and late winter (P < 0.0001). Plasma from bats collected in early winter had higher BA than in late winter (P = 0.047) and autumn (P < 0.0001) (Fig 7) BA varied significantly in P2 ($H_{2, 94}$ = 6.3, P = 0.043) with higher values in autumn than late winter (P = 0.046), but similar to levels in the summer (P > 0.999). BA varied between periods ($H_{6, 185}$ = 120.3, P < 0.0001) but only in summer with higher values in P1 (P < 0.0001).

Effect of acute stress stimuli

Compared to basal levels, SOD activity was greater after 6 h of immobilization stress during summer (t_8 = 3.06, P = 0.015) and during early winter (t_{21} = 3.19, P = 0.0044) and GPx activity was increased after 12 h of immobilization during summer (t_8 = 2.88, P = 0.021; Fig 8) in P1. No increase in carbonyls was detected in early winter (t_7 = 1.53, P = 0.17); however, carbonyl

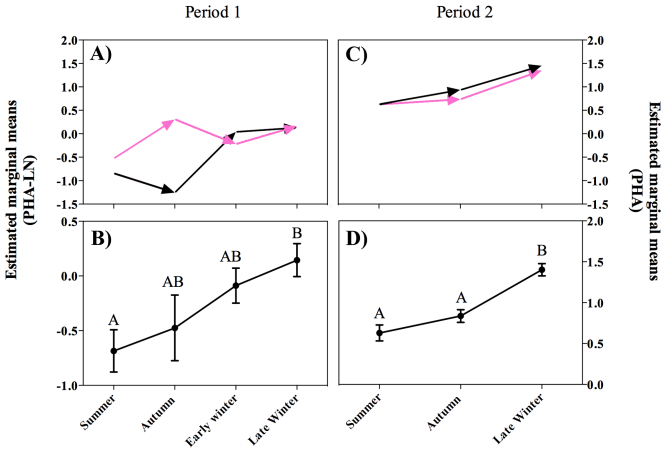

Fig 6. Seasonal swelling response after 24 hours of PHA injection. A) Data separated by sex from P1. B) Combined data from P1 (estimated marginal mean ± SE). C) Data separated by sex from P2. Pink arrows represent seasonal variation in the swelling response in females; black arrows represent seasonal changes in swelling response in males; D) Combined data from P2 (estimated marginal mean ± SE); different letters indicate significant differences among seasons.

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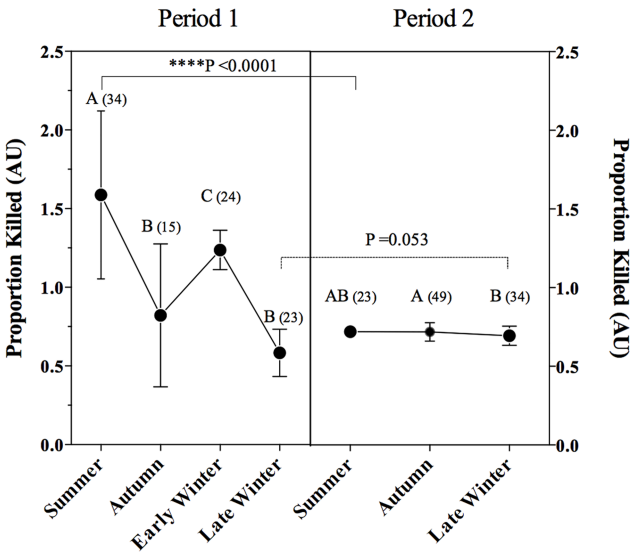

Fig 7. Bactericidal activity of plasma. Samples correspond to seasons within periods 1 (mean ± SD), and 2 (median ± interquartile range), as revealed by ANOVA analysis with Welch correction and Kruskal-wallis analysis, respectively. Different letters indicate significant differences between seasons within a period, asteisks indicate significant differences between the same season of different periods, as revealed by Kruskal-Wallis post-hoc analysis. Numbers in parenthesis indicate sample sizes.

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content in summer after 6 h of stress during the summer (t_7 = 2.82, P = 0.025) BA increased during summer after 12 h of stress (t_{17} = 2.82, P = 0.025; Fig 9). In P2, CAT activity was greater in late winter (t_{11} = 2.696, P = 0.021) and GPx activity was greater in summer (t_{17} = 2.830, P = 0.012) and lower in late winter (t_{16} = 2.629, P = 0.018; Fig 8) compared to basal levels. Carbonyl content did not increase following immobilization in summer, but it increased in autumn after 6 h of stress (t_{15} = 2.701, P = 0.016) and in late winter after 12 h of stress (t_{17} = 2.285, P = 0.035; Fig 9). BA increased during summer after 6 h of stress (t_{17} = 5.47, P <0.0001; Fig 9).

Principal component analysis (PCA): Inter-correlation between variables

The PCA made on the basal values of the physiological markers, the first four components explained 83% of the variance, independent of groups (D-PCA) in P1. PC1 was correlated with $d\overline{\digamma}_a$ (+) and %H (+), PC2 with sex (+) and reproductive status (rep) (+), PC3 with SOD

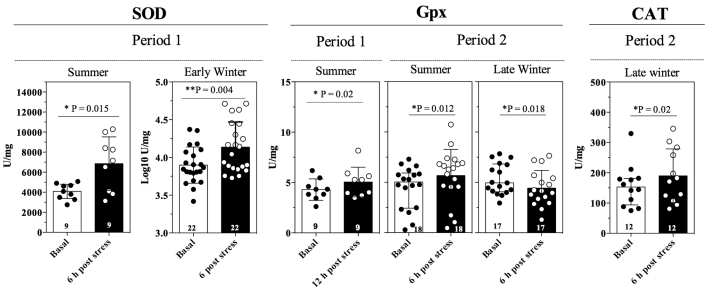

Fig 8. Acute stress stimuli impact on physiological markers. Change in SOD, CAT and GPx activity (mean ± SD). Asterisks indicate significant differences in the response between basal and post-stress levels based on Student's t-tests. Bold numbers inside bars indicate sample sizes.

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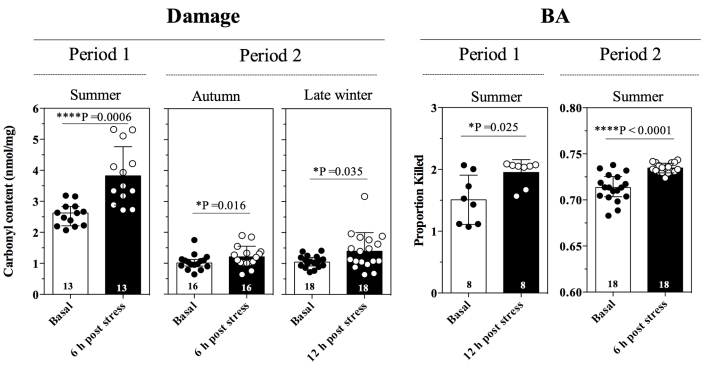

Fig 9. Acute stress stimuli impact on physiological markers. Change in carbonyl content and BA (mean ± SD) Asterisks indicate significant differences in the response between basal and post-stress levels based on Student's t-tests. Bold numbers inside bars indicate sample sizes.

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(+), and PC4 with CAT (+), PHA (+) and BA (-) (Table 1). When plotting PC1 against PC2 (Fig 10A), bats had higher CAT and SOD activity levels, and higher hematocrit percentage and BA at high T_a. When T_a was low, bats had higher swelling responses, but in particular reproductive males presented the highest PHA index. The between-group principal component analysis (BG-PCA) explained 92% of the variance within the first two components, which pointed out the variables responsible of separating the groups in PC1 (+CAT, +H%, +$d\overline{T}_a$, +sex) and PC2 (+SOD, +PHA, +rep) (Table 1). The biplot (Fig 10B) revealed a clear separation among groups, except for early and late winter, which displayed a spliced area. T_a was at its highest level in summer and bats were mainly characterized for having high BA and H% during this period. Bats had their highest SOD and CAT activity in autumn, while during early winter they still retained some CAT activity. All bats in late winter had low SOD and CAT activity but high PHA response, particularly male bats. Although low temperatures were related with low AEA, protein oxidative damage seemed not to play an important role (coefficient lower than 0.5) in the model of undisturbed bats. The D-PCA of post stress data (6 hours) (Table 1) explained 78% of variance within the first four components (Table 1). PC1 was correlated with SOD6 (+), sex (-) and rep (-), PC2 with CAT6 (+) and SMI (-), PC3 with BA6 (+), and PC4 with carbonyl6 (-) (Table 2, supplementary material). Stress response was highly dependent of bat sex and reproductive state, with non-reproductive females having higher SOD and CAT activity, as well as an increment in BA and carbonyls but not as strong, suggesting that in general they are capable of mounting an efficient defense against unknown sources of stress, especially when they have low body conditions (Fig 10C). The post stress data (6 hours) BG-PCA explained 91% of the variance within the first two components. PC1 (+Carbonyl6, +BA6, +SMI, +rep) suggest that reproductive bats with good body condition had higher protein damage and cellular immune response, and PC2 (+SOD6, +CAT6, -sex) indicates that females had higher antioxidant defenses (Table 1). The biplot (Fig 10D) reveals an even further group separation compared to the basal data BG-PCA. During autumn and summer, bats mounted a high CAT and SOD response, but during summer bats had increased BA and protein damage. The area plot for early winter expanded, so it overlapped with late winter and with summer; this divergent stress response was sex dependent, with females being more prone to have high antioxidant defenses and high protein damage in contrast to males.

Basal values explained 81% of the variance in the first four components of D-PCA in P2 (Table 2). PC1 was correlated with CAT (+) and $d\overline{T}$ (+), PC2 with $\hat{W}_n$ (+) and SMI (+), PC3 with SOD (+), and PC4 with GPx (+) and PHA (+) (Table 2). When plotting PC1 against PC2 (Fig 10E), bats had higher antioxidant activity levels (especially CAT) with high T_a, while

Table 1. Component correlation matrix of principal component analysis (PCA) used to determine relationships between physiological variables in the first period of sample collection.

Type	PCA	Variable	PC1	PC2	PC3	PC4
Basal	D	Eigenvalues	234.605	187.294	143.655	0.990458
		Percent of variance	29.33	23.41	17.96	12.38
		Cumulative %	29.33	52.74	70.70	**83.08**
		BA	0.357	0.251	0.307	**-0.533**
		SOD	0.001	0.045	**0.736**	-0.271
		CAT	0.361	0.235	0.284	**0.515**
		PHA_24	-0.196	0.270	0.357	**0.580**
		%H	**0.529**	0.009	-0.214	0.120
		Sex	-0.283	**0.569**	-0.207	-0.078
		Rep	-0.098	**0.673**	-0.181	-0.140
		dTm	**0.579**	0.174	-0.184	0.033
	BG	Eigenvalues	397.53	338.96		
		Percent of variance	49.69	42.37		
		Cumulative %	49.691	**92.061**		
		BA	0.327	0.299		
		SOD	0.201	**-0.426**		
		CAT	**0.446**	-0.247		
		PHA_24	-0.125	**-0.524**		
		%H	**0.488**	0.079		
		Sex	**-0.447**	0.233		
		Rep	-0.001	**0.533**		
		dTm	**3.000**	0.220		
6h stress stimuli	D	Eigenvalues	22.64	170.31	125.01	100.37
		Percent of variance	28.30	21.29	15.63	12.55
		Cumulative %	28.30	49.59	65.21	**77.76**
		BA6	0.1855	0.05007	**0.692**	0.5319
		SOD6	**0.4847**	0.23	-0.043	0.4012
		CAT6	0.1922	**0.6893**	0.1077	-0.2285
		Carbonyl 6	0.2393	0.01307	0.4773	**-0.6999**
		SMI	0.2466	**-0.6607**	0.2744	-0.0659
		Sex	**-0.5508**	-0.001836	0.1636	0.09569
		Rep	**-0.5218**	0.181	0.4217	0.02447
	BG	Eigenvalues	402.16	312.10		
		Percent of variance	50.27	39.01		
		Cumulative %	50.27	**89.28**		
		BA6	**0.4965**	0.2353		
		SOD6	-0.1217	**0.5271**		
		CAT6	-0.08278	**0.4504**		
		Carbonyl6	**0.5327**	0.04164		
		SMI	**0.5142**	0.06826		
		Sex	-0.1432	**-0.5606**		
		Rep	**0.404**	-0.3786		

Note. PCA analysis was performed as Disregard PCA analysis (D), and between groups PCA analysis (BG). Loadings highlighted in bold and underlined represent the highest absolute value of the variable when considered different PCs. BA = Bactericidal activity, BA6 = BA after 6 h stress, SOD = Super oxide dismutase, SOD6 = SOD after 6 h stress, CAT = catalase, CAT6 = CAT after 6 h stress, Gpx = Glutathione peroxidase, GPx6 = GPx after 6 h stress, %H = percent of hematocrit, rep = reproductive state, SMI = scaled mass index, Carbonyl6 = carbonyl content after 6 h stress, dTm = mean daily temperature, PHA_24 = Phytohaemagglutinin 24 h index.

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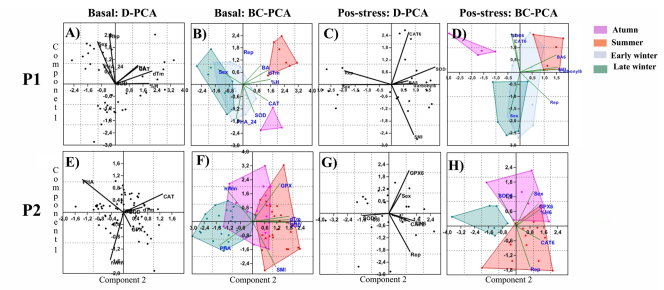

Fig 10. Graphic representation of principal component analysis plotting PC1 against PC2. A) Period 1 basal disregard-PCA, B) Period 1 basal between groups-PCA, C) Period 1 six hours Post-stress disregard-PCA, D) Period 1 six hours Post-stress between groups-PCA, E) Period 2 basal disregard-PCA, F) Period 2 basal between groups-PCA, G) Period 2 six hours Post-stress disregard-PCA, H) Period 2 six hours Post-stress between groups-PCA.

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during windy nights GPx was the main antioxidant defense followed by SOD. The swelling response tended to be greater on low T_a and windy nights, and similarly to P1, protein oxidative damage did not to have an important effect during basal conditions. The BG-PCA explained 77% of the variance within the first component (Table 2), with SOD (+), CAT (+), dŦ (+), and PHA (-), as the variables mainly responsible for separating the groups. The biplot (Fig 10F) showed a separation between summer and winter, with autumn between these seasons. During summer, bats had high SOD, CAT and GPx activity and a greater SMI, whereas in winter bats had strong swelling responses and low SOD, CAT and GPx activity. 82% of the variation in the post stress (6 hours) response was explained by the first four components of the D-PCA data: PC1was correlated with H%6 (+), PC2 with GPx6 (+) and rep (-), PC3 with

Table 2. Component correlation matrix of principal component analysis (PCA) used to determine relationships between physiological variables in the second period of sample collection.

Type	PCA	Variable	PC1	PC2	PC3	PC4
Basal	D	Eigenvalues	210.88	15.10	107.92	0.97
		Percent of variance	30.13	21.57	15.42	13.87
		Cumulative %	30.13	51.70	67.12	**80.98**
		SOD	-0.018	0.350	**0.761**	0.267
		CAT	**0.561**	0.161	0.089	0.309
		GPX	0.164	0.305	-0.584	**0.598**
		PHA	-0.361	-0.308	0.188	**0.643**
		SMI	0.311	**-0.536**	0.115	0.191
		dTm	**0.607**	0.118	0.151	-0.160
		nWm	-0.250	**0.602**	-0.001	-0.011
	BG	Eigenvalues	541.89			
		Percent of variance	77.41			
		Cumulative %	**77.41**			
		SOD	**0.430**	-0.015		
		CAT	**0.429**	0.026		
		GPX	0.323	0.524		
		PHA	**-0.392**	-0.325		
		SMI	0.273	-0.614		
		dTm	**0.428**	0.073		
		nWm	-0.340	0.487		

(*Continued*)

Table 2. (Continued)

Type	PCA	Variable	PC1	PC2	PC3	PC4
6h stress stimuli	D	Eigenvalues	195.15	109.88	0.98	0.91
		Percent of variance	32.53	18.31	16.40	15.16
		Cumulative %	32.53	50.84	67.24	**82.41**
		BA6	0.186	0.050	**0.692**	0.532
		SOD6	**0.485**	0.230	-0.043	0.401
		CAT6	0.192	**0.689**	0.108	-0.229
		Carbonyl 6	0.239	0.013	0.477	**-0.700**
		SMI	0.247	**-0.661**	0.274	-0.066
		Sex	**-0.551**	-0.002	0.164	0.096
		Rep	**-0.522**	0.181	0.422	0.024
	BG	Eigenvalues	388.20	211.80		
		Percent of variance	64.70	35.30		
		Cumulative %	64.70	**100.00**		
		BA6	**0.497**	0.235		
		SOD6	-0.122	**0.527**		
		CAT6	-0.083	**0.450**		
		Carbonyl6	**0.533**	0.042		
		SMI	**0.514**	0.068		
		Sex	-0.143	**-0.561**		
		Rep	**0.404**	-0.379		
12h stress stimuli	D	Eigenvalues	219.90	163.34	119.06	107.31
		Percent of variance	27.49	20.42	14.88	13.41
		Cumulative %	27.49	47.90	62.79	**76.20**
		SOD 12	**-0.668**	0.374	0.491	0.098
		CAT 12	0.251	0.396	**0.764**	0.187
		GPX 12	-0.034	0.247	-0.277	**0.775**
		CARBONYL 12	**0.586**	0.240	0.062	-0.510
		SMI	**0.687**	0.319	0.018	-0.045
		H% 12	**0.592**	0.341	-0.343	0.235
		Sex	**-0.712**	0.407	-0.362	-0.307
		Rep	-0.124	**0.914**	-0.190	-0.132
		Eigenvalues	537.466	262.534		
		Percent of variance	67.183	32.817		
		Cumulative %	67.183	**100.000**		
		SOD 12	0.293	**0.453**		
		CAT 12	**0.392**	-0.258		
		GPX 12	0.163	**0.571**		
		CARBONYL 12	**0.402**	0.223		
		SMI	**0.431**	-0.014		
		H% 12	0.250	**-0.503**		
		Sex	**-0.375**	0.306		
		Rep	**0.428**	0.075		

Note. PCA analysis was performed as Disregard PCA analysis (D), and between groups PCA analysis (BG). Loadings highlighted in bold and underlined represent the highest absolute value of the variable when considered different PCs, BA6 = BA after 6 h stress, BA12 = BA after 12 h stress, SOD = Super oxide dismutase, SOD6 = SOD after 6 h stress, SOD12 = SOD after 12 h stress, CAT = catalase, CAT6 = CAT after 6 h stress, CAT12 = CAT after 12 h stress, Gpx = Glutathione peroxidase, GPx6 = GPx after 6 h stress, GPx12 = GPx after 12 h stress, %H = percent of hematocrit, %H12 = %H after 12 h stress, rep = reproductive state, SMI = scaled mass index, Carbonyl6 = carbonyl content after 6 h stress, dTm = median daily temperature, nWm = night median wind speed, PHA = Phytohaemagglutinin 24 h index.

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CAT (-) and sex (+), and PC4 with SOD (+) (Table 2). The biplot (Fig 10G) showed that non-reproductive males enhanced their GPx activity during the stress response, while lactating females enhanced their CAT activity, but bats that presented high SOD activity had low CAT or GPx activity. The post stress BG-PCA data explained 100% of the variance in PC1 (+CAT6, +GPx6, +%H6) and PC2 (+SOD6, -rep, +sex; Table 2). The biplot (Fig 10H) revealed that bats from each season had distinctly different stress responses. During autumn, non-reproductive males increased their SOD and CAT activity, while during summer bats (that in basal conditions had high SOD, CAT and GPx activity) only enhanced their CAT activity, particularly lactating females., Antioxidant activity increased in winter but a lesser extent compared to autumn and summer. As in P1, variation in protein oxidative damage did not correlate strongly with other parameters in the 6 hours post-stress model. 76% of variance in the post stress (12 hour) model was explained by the first four components of the D-PCA analysis: PC1 was correlated with SOD12 (-), Carbonyl 12 (+), SMI (+), H%12 (+) and sex (-), PC2 with rep (+), PC3 with CAT (+) and PC4 with GPx (+) (Table 2). The biplot (Fig 1) showed that after 12 h of stress, bats (especially lactating females and in good body condition) increased their AEA and protein carbonylation; however, there was a significant negative correlation between SOD and Carbonyls in PC1. The post stress BG-PCA data explained 100% of the variance in PC1 (+CAT12, +Carbonyl12, +SMI, +sex, +rep), and PC2 (+SOD12, +GPx12, +H%12) (Table 1). The biplot (Fig 11J) showed that after 12 h of stress, bats had a similar response in summer and autumn, in contrast to bats from winter. Lactating females with high SMI increased CAT, SOD and GPx activities and protein carbonylation in summer. Surprisingly, bats in winter had low CAT, SOD and GPX activities and a very low increase in carbonylation.

Discussion

The study of physiological changes from basal to post-stress levels at different times of the year has largely been neglected; as a consequence, the few available published studies report contradictory results [9,54,55]. We propose that such discrepancies arise from a heterogeneous sampling method, and that just as basal levels set points are dynamic through the year, stress response also varies across the annual cycle. Therefore, animals would be more susceptible to unpredictable sources of stress at certain times of the year. We evaluated basal immunocompetence and redox state change during autumn, summer and winter and assessed the effect of acute stress. Our results showed that the basal immune response was an important physiological response through the year, although the particular type of response depended on the time of the year.

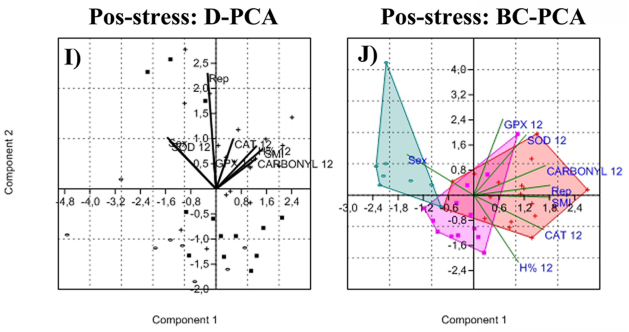

Fig 11. Graphic representation of principal component analysis, plotting PC1 against PC2. I) Period 2 twelve hours Post-stress disregard-PCA, H) Period 2 twelve hours Post-stress between groups-PCA.

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PHA response and plasma bactericidal activity are functionally different immune response measurements. Plasma BA is a constitutive response related to humoral resistance to extracellular infections, while the PHA response is an induced, mostly cellular, immune response related to resistance to intracellular infections [90]. However, recent debate has questioned the interpretation of PHA injection-site swelling as an index of T cell-mediated immunocompetence, and it has been reported that other leukocytes (basophils, neutrophils, and eosinophils) may be primarily responsible for localized vasodilatation, infiltration and edema, resulting in inflammation of the PHA injection site [73,74,91]. Therefore it has been recommended that tissue biopsies from the injected areas should be recollected in order to determine specific leukocytes migration to the injected site. It has been reported that basophils are the main leukocytes responsible for inflammation after 6 h post injection [92]; while neutrophils, lymphocytes, and macrophages peak between 12 and 24 h post injection [91–94]. We also know that the number of lymphocytes present pre-treatment positively affects the number of lymphocytes infiltrating into tissue [95], suggesting that the levels of basal leukocyte populations is determines the intensity of the response when it is presented with a novel antigen. This is also supported by the findings of decreased heterophil number in peripheral blood due to rapid and intensive tissue infiltration [96]. Similarly, striped hamsters (*Cricetulus barabensis*) showed a tendency for lower neutrophil proportions in peripheral blood 24-hr after PHA stimulation [94]. For this reason, PHA inflammation can still be an overall indicator of the immune response of organisms when presented with a novel antigen, even without assessment of the specific cellular population migrating to the site.

Overall, our results were concordant with other literature reports, which have shown that variation in immune cell abundance and distribution on a seasonal basis is common [46]. The PHA index and BA were found to be negatively correlated during P1, which agrees with other literature reports [22]. The magnitude of the PHA response was not significantly different between sexes through the year in both periods examined. The PHA response only differed briefly during P1 at 6 h after injection, but the difference between sexes disappeared after 12 and 24 h. The magnitude of the PHA response was generally greater during winter, while plasma BA was higher during summer [46,47]. A possible explanation is related to the low energy requirement needed to induce the PHA response [97,98], since bats tend to forage less during the winter, achieving lower daily rates of energy intake and thus predisposing animals to a recurrent use of torpor [57]. At the same time, the high metabolic cost of prey digestion further limits animals net energy intake [99]. Furthermore, in both in-between and disregard PCA analyses, the magnitude of the PHA response was positively correlated with at least one enzymatic antioxidant activity in different basal conditions: with CAT and SOD in P1, and with GPx in P2.

Part of the immune response relies on immune cells that kill pathogens by releasing pro-oxidant compounds. Phagocytosis stimulates the so-called "respiratory burst" which results from NADPH oxidase activation, an enzyme normally inactive in resting cells. NADPH oxidase produces the superoxide free radical (O_2^-). These reactive oxygen species can destroy microorganisms or other foreign matter [100,101]. Hence, immune cells are particularly sensitive to oxidative stress, since ROS generated outside the immune cells can affect the integral membrane function, including the cell-mediated immune reaction involving phagocyte membrane NADPH oxidase which leads to depressed immunocompetence [100]. Furthermore a continually activated immune response (i.e. chronic inflammation) may cause extensive tissue oxidative damage due to the consequent sustained ROS increase [102]. Therefore, the positive correlation between PHA response and the enzymatic activity found in our study, as well as the low carbonyl presence during winter, suggests that bats are able to maintain their redox balance while maintaining a high inflammation index.

Plasma BA during P1 was generally (D-PCA) positively related with CAT activity; however, bats with high SMI scores increased BA after the 6-hour stress. Our results agree and support the hypothesis that after short acute stress the immune response can be enhanced [55,90,103–107]. However, this occurred only during summer, when more energetic demanding processes are taking place, and no changes in BA were found during winter or autumn. This suggest that intensity of the humoral immune response to stress is highly dependent on the time of the year when it is being measured, and might explain the discrepancies found in several reported results on wild animal [54,108]. After the 12-h stress episode, BA was no different from basal levels, which suggests that after prolonged stress investment in an enhanced humoral immune response is reduced, but maintained at basal levels.

In regards to AEA defenses, our results showed that SOD and CAT in particular play an important role in *M. vivesi* homeostasis maintenance through the year, and that their activity was mostly related to environmental cues. The fact that protein damage had a low coefficient when testing the PCA model suggests that bats maintain redox balance and have minimum systemic damage.

Seasonal variation in the physiological variables was clearly apparent in our analyses as shown by dissimilar physiological responses presented by bats across seasons, which denoted their ability to modulate their defenses according to environmental changes and time of the year, partially supporting our hypothesis. However, winter was apparently not the most adverse stressful season as we predicted based on the low levels of AEA and carbonyl concentration measured in both periods reflecting low oxidative damage. This finding directly contrasted with reports describing high oxidative damage and high AEA during winter in birds [109]. As mentioned before, strong winds, strong surf and low T_a during winter may limit foraging in fish-eating Myotis promoting the use of torpor [57]. As we only sampled active bats, it is impossible to know if their low AEA activity is related to the enzymatic deployment during the respiratory burst that occurs during torpor arousal [110,111]. Either way, redox balance appeared to be undisturbed during this season, and stress did not seem to have any detrimental effect or change on their basal physiological parameters. Meanwhile, autumn was the season in which bats presented their highest SMI and AEA defenses and, as predicted, this season acted as a physiological buffer between winter and summer. Bats in summer also presented high AEA, which was strongly correlated with the elevated seasonal temperatures, to which we know *M. vivesi* are highly sensitive [56]. Moreover, bat reproductive state also played a major role in AEA response during this season. ROS production increases during catabolism, immune challenges, and periods of environmental stress [112]. Therefore, activities that increase metabolic demands, such as reproduction, could lead to greater ROS production, thereby imposing a higher demand for antioxidant synthesis or transport and might require the use or maintenance of repair mechanisms to avoid oxidative damage accumulation [112]. This effect was probably reflected in bat´s response to unpredicted acute stress. Bats generally responded to acute stress (both after 6 and 12 h) by enhancing their AEA activity, especially during summer and autumn, and to a lesser extent in early winter, but it was during summer when protein damage increased after stress episodes. This suggests that when energetic stress increases as a result of the simultaneous occurrence of multiple physiological challenges (i.e. humoral immune response, AEA response, thermoregulation demands due to high temperatures, and lactation) higher levels of oxidative damage may accumulate due to imbalances in oxidative stress and the capacity to mitigate such damage.

Female mammals tend to invest more energy into parental care than males, and lactation is their most energetically demanding parental endeavor [113,114]. As a consequence, it has been hypothesized that oxidative stress increases during lactation [114]. Lactating Mongolian gerbils (*Meriones unguiculatus*) had lower antioxidant defense and greater protein damage than non-reproductive females [115]. Lactating red squirrels (*Tamiasciurus hudsonicus*) had

higher protein and oxidative damage, more energy demand and lower antioxidant defenses than non-reproductive females [103]. However, when lactating squirrels were supplemented with abundant food resources, they presented elevated antioxidant protection and reduced plasma protein oxidation relative to squirrels on natural (low) food resource [116]. These reports coincide with our results. During summer, most females were lactating, and even so they had increased AEA activity, concurring with a high SMI and hematocrit percentage, suggesting that high body condition and abundant resources available enable them to balance oxidative damage during this period [57,117]. However, if stress is extended for a prolonged time (e.g. 12 h), oxidative stress increases and it begins to produce substantial damage. Our study did not evaluate the degradation and replacement mechanism of oxidized proteins, so it is not possible to determine if protein damage generated after stress was accumulated or not. Even so, the antioxidant defense enhancement presumes an effective physiological response to maximize fitness. Therefore, our results suggest that *M. vivesi* is capable of modulating its physiological mechanism to maintain homeostasis through the year, and that individuals are highly resilient to unpredictable stress. If enough food resources are available bats would be able to oppose oxidative stress and maintain fitness even during periods of high energy demand such as lactation. These findings support the predictions of the allostasis hypothesis. Therefore, taking into account the time of the year while interpreting stress response its and integral part.

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CAPITULO II:

Glucocorticoids seasonal variation and its relation to immunocompetence and oxidative stress in an endemic insular bat

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Introduction

One of the most common tools in conservation physiology is the rapid assessment of environmental stress via the measurement of glucocorticoid 'stress' hormones. These steroid hormones are ubiquitous in vertebrates, and occur at low (baseline) levels in all individuals (1). These hormones are usually measured in plasma (2) and in other biological samples (saliva, hair, urine and feces) (3). In many cases when animals experience inclement weather (4) or risk of predation (5,6), glucocorticoids (GCs) increase in circulation and, subsequently, in the feces. Conservation physiologists often experimentally induce mild stress (capture and handling) to assess individuals' ability to cope with an unpredictable novel environment and survival at the long run (7–11).

GCs also play important roles in non-stressful situations by mediating energy balance through the stimulation of glycogenesis, proteolysis and adipose tissue formation (12–14). GCs are known to vary with predictable changes in the environment on a daily (day/night periods, high tide/low tide periods) or seasonal basis (15–17), and they change with life history traits such as reproduction (18,19), migration (20–22), or social interactions.

The assessment of GCs in plasma of wild animals can prove difficult in many cases. Circulating hormone levels increase within 3-5 minutes after capture in birds and small mammals (2), and obtaining plasma samples within this time frame is sometimes difficult in many field settings. Furthermore, plasma provides hormone concentration at a single point in time and it might be not representative of long-term hormone exposure levels for GCs that exhibit both regular and erratic changes with time (23). Therefore, the use of non-invasive techniques as fecal sampling has become a preferable measure of GCs (3,23–25) in the field. However, interpretation of GCs in fecal samples also comes with its own set of difficulties. When metabolic rate, food intake and hormone metabolite excretion of the focal animal is not known, results can potentially be miss-interpreted (26). In cases when such information is

missing, GCs data could potentially relay on other physiological stress biomarkers to completely an accurately assess animal's condition and response to stress.

GCs excretion and immune systems are often negatively correlated: high levels of GCs often down-regulate immune reactivity (27,28). Thus, there appears to be a trade-off between GC stress response and immunity in that a high activity of the firts system constrains the activity of the second (29). It has been experimentally shown that acute stress (i.e short-term stress from minutes to hours) boosts the immune response by re-locating the cellular components (leukocytes) on the skin (30,31). On the other hand, chronic stress (i.e long-term stress from days to months) causes immunosuppression by shifting the cytokine balance from a TH1 profile (pro-inflammatory) to a TH2 profile (anti-inflammatory) hence decreasing the number of circulating leukocytes (30). However, whether stress stimulates or inhibits immune response also depends on the immune parameter assessed. For example chronic exposure to corticosterone reduced the production of antibodies of barn owl nestlings (*Tyto alba*) but it did not significantly affect the constitutive innate immunity (32).

An associated cost of the immune system and GC stress response is the production of reactive oxygen species (ROS) (33,34). ROS are highly reactive molecules which are ubiquitous in all aerobic organisms (35). However, the relationship between GCs and oxidative stress is still not clear. A negative relationship between plasma GCs and mitochondrial superoxide was found for grey partridges (*Perdix perdix*), which might highlight the regulative role of GCs in the context of resistance to oxidative stress (29). Nevertheless, there have been reports of GCs inducing oxidative stress. For example, dexamethasone (a synthetic GC), significantly enhanced ROS levels in Sprague–Dawley rats hippocampal slice cultures (36). In the common lizard (*Lacerta vivipara*), high carotenoid (an antioxidant) coloration was related to high corticosterone concentrations however, this enhancement disappeared under food restriction, and corticosterone had a negative effect on

carotenoid-based coloration and induced oxidative stress (37). Similarly, nestlings of barn owl (*Tyto alva)* showed a significant reduction in resistance to oxidative stress after 12 days of implantation of corticosterone-releasing pellets (32).

Here we tested the relationship between fecal cortisol metabolites and different antioxidant enzymes (by measuring SOD, CAT and GPx), oxidative damage (by measuring protein carbonylation), and immune parameters (humoral response by measuring bactericidal activity (BA) and inflammatory response by injecting Phytohemaglutanin (PHA)) in the fishing-eating Myotis (*Myotis vives*i, Menegaux, 1901), a bat endemic to the islands of the Gulf of California, Mexico. Islands where this bat roost are exposed to seasonal changes in ambient temperature (T_a), ranging from 45ºC in summer to 5ºC during winter (38). Bats roost under rocks during daytime, which provide partial isolation from T_a (39). Lactation in this bat occurs during summer, and during winter these bats use torpor (39), two factors that are known to influence GCs levels (40–43). We used this bat as a model to describe the metabolite cortisol profile and its response to acute stress (23,44–48), and the changes induced on antioxidant and immune response by acute stress (endogenous GCs inducement by movement restriction stress up to 24 h) and by a stronger and prolonged stress induced by injection of exogenous ACTH, monitoring up to 48 h in an experimental procedure.

We hypothesized that bats will have an enhanced immune response under an acute stress, and that GC concentration would be positively related to antioxidant activity, with no generation of protein damage under acute stress. While under prolonged stress (48 h) GC would have a negative association with the immune response.

Material and methods

Study site

This study was conducted in Partida Norte Island (28º52'30''N, 113º21'7''W), a 1.4-km^2 island located in the midriff region of the California Gulf, Mexico (49). This island holds the largest known colony of fish-eating Myotis (~8,000 adults)(50). Fieldwork was conducted for five days each during summer (July 2014), fall (October 2014), and early winter (December 2014), and late winter (February 2015). These periods include the months when lactation occurs (July), when bats enter torpor (December and February), and a transition period (October). Sample collection during spring was not conducted because the population is composed almost exclusively of pregnant females (51) and we consider that our manipulations might be too invasive for these animals.

Animal handling and samples collection

All bats captured were sampled for blood and fecal samples, and record of weight and forearm length was taken. We captured only adult females for the study between 6:00-7:00 am directly from their roost sites under the rocks. Adults were differentiated from young individuals by examining their ossification index. Accordingly, we exposed the wing of each individual to transillumination using a headlight; in young individuals, the cartilaginous zone of the long phalanges is visible because less mineralized tissue allows more light to pass through and thus appears lighter than bone. When they reach adulthood, epiphyseal plates eventually close and are no longer visible to the unaided eye. Fur color was also an indicator of age; adults had brown-gold color while youngs had gray color (52). Bats were placed in individual small cotton bags, where the acute stress stimuli consisted of their immobilization within the bag. Blood samples were taken for antioxidant activity, carbonyl determination and bactericidal activity (BA). A first (within 30 min of capture) blood sample (150 μl) for antioxidants

analysis was obtained by bleeding bats from the right forearm vein. This first sample was considered as basal, as enzymatic antioxidants are primarily transcriptionally regulated, and BA activity in plasma has been shown no to change after 1:30 h of capture in other species of bats (53). After the first blood sample, bats were injected subcutaneously in the footpad with Phytohemaglutanin (PHA) to assess cellular immune response (described further in corresponding section). Bats were maintained in the bag for 6 h after capture and a second blood sample (150 μl) was drawn from the left forearm vein. Blood was drawn by venipuncture with a hypodermic needle (27Gx 13mm BD), collected with heparinized capillary tubes, and placed in 1.5 mL eppendorf tubes. All samples were kept in ice during collection and handling in the field, and plasma and erythrocytes were separated by centrifugation at 6000 rpm (Digital ZipSpin Centrifuge LWScientific) and stored in liquid nitrogen in 0.2 mL tubes and frozen. Samples were transferred from liquid nitrogen to dry ice and shipped to the laboratory where they were stored at -80ºC until their analysis.

We collected fecal samples directly form the cotton bag of each bat captured. Bags were examined every 30 min for defecations and we registered its time of collection. Samples were stored in liquid nitrogen until transportation to the laboratory, were they were kept at –20°C until their metabolite extraction (≤ 6 months) at the Physiology Department of Centro de Investigación y de Estudios Avanzados, in Mexico City.

Bats were feed with shrimps twice while in captivity. The first meal was provided approximately 5 to 6 h after capture, and a second meal was provided 12 h after capture. Intestine transit time (ITT) was measured by using blue vegetal pastry dye in one of the meals. The time the dyed food took to be digested was recorded. ITT (mean ± SD) was of 9.6 ± 2.5 h (n = 7) during autumn, 9.8 ± 2.1 h (n = 8) during early winter, 10.6 ± 4.63 h (n = 10) during late winter, and 8 ± 3.5 h during summer (n =19). As the shortest transit time found was 6 h, we considered all feces deposited before that time as basal samples. Time of collection of

basal sample after capture were 0.64 ± 0.7 h (n = 7) during autumn, 1.8 ± 0.9 h h (n = 8) during early winter, 1.7±1.4 (n = 10) during late winter, and 1.6 ± 1.1 h (n = 19) during summer. According to this, in order to compare stress reactivity between seasons we grouped samples as follow: Stress reactivity 1 (SR1) were the samples collected between 6 and 14 h after capture, and stress reactivity 2 (SR2) were the samples collected between 15 and 24 h. After 24 hours, bats were returned to the place where they were captured.

The study was conducted in strict accordance with the recommendations and permits approved by Mexican authorities (permits 13/13 from Secretaría de Gobernación and 01947/13from Dirección General de Vida Silvestre to LGHM). All sampling procedures and experimental manipulations were performed according to the Principles of the Mexican Official Ethics Standard 062-ZOO-1999. No other approval was required to conduct the study, as there is no IACUC/animal ethics board at our institution.

Fecal Glucocorticoid Metabolites (FCM) assay

The protocols that we used to extract (47,54) GC metabolites from feces have been previously used and validated to detect the activation of the HPA axis in response to stress in different wild mammals. We modified the methods in order to adjust to the low mass of samples (≈ 0.05 g). Accordingly, we dried out the samples at 65°C in a scientific oven (Precision Scientific 25EM), pulverized them, and weighed each sample. We placed the samples in 3 mL glass tubes, 600 µL of ethanol 100% were added, and then they were vortexed for 1 min every 30 min for 3 hours and incubated at 4ºC for 12 hours. Samples were vortexed again for 3 min and centrifuged at 4ºC for 15 min at 3000 rpm. The supernatant was decanted in 1.5 mL tubes and samples were centrifuged again at 4ºC for 10 min at 10,000rpm. The process was repeated until all solid matter was removed. Samples were dried in a hot plate with nitrogen flow. Samples were then re-suspended by adding 160 µL of ethanol 100%, and kept at –24°C until

radioimmunoassay (RIA). Fecal extraction efficiency was as measured by the recovery of ^{125}I-cortisol (66.4 ± 1.1, mean ± SE, $N = 6$).

We quantified cortisol concentrations in the samples with a solid phase ^{125}I RIA method using cortisol CORT-CT2 CIS kits (Bio Internacional® B.P. 32-F91192 GIF-SUR-YVETTE CEDEX/France), following the protocol for saliva samples as indicated by the manufacturer. The calibration range for the assay was 0–2000 nmol l^{-1}. The samples were incubated for 30 min at 37°C radioactivity measured using a Packard Cobra II® (Packard Cobra II, A Canberra Co. Meriden, CT) scintillation counter for gamma radiation. The kit presents a low cross-reactivity with corticosterone (2.5%) and cortisone (2.2%). We assessed all the extracts with one replica (no dilutions were made in order for samples to enter within the standard curve of the kit, as cortisol levels on this species were unknown), in a total of two assays. Intra- and inter-assay coefficients of variation were 0.73% and 4.92%, respectively. The option in the linear regression test for testing differences between lines (differences in slopes) was performed. The calculation followed the methodology proposed by Zar (1984), which is equivalent to an analysis of covariance (ANCOVA) that is used to compare more than two lines. There were no significant differences ($F_{2,9} = 0.0733$, $P = 0.9298$, $N = 2$) between the slopes of a serial dilution curve of pooled bat fecal extracts and the slope of the standard curve. The slope of standards spiked with diluted fecal extract exhibiting high accuracy ($B = -27.28$, $R^2 = 0.837$ $P = 0.0038$), indicating that the assay reliably measures FCM across its range of concentration. The minimum detection limit for this assay was 14 ng of GC per 50 µg of bats feces.

Body condition determination

Ecologists have measured body condition as a non-destructive method to estimate nutritional state and provide a snapshot of an animal's physiological state (55). Here, body condition was

determined for each bat with two methods in order to determine if it was related to their GC response. First, we used the Scaled Mass Index (SMI) (56) which relies on measures of body mass and linear measures of body size to calculate a condition index with the following formula:

$^{Mi}= Mi [L_0/L_i]^{bSMA}$

where Mi is the weight (g), L_i is the forearm length (mm), L_0 is the forearm arithmetic mean, bSMA is the scaled exponent estimated using online software (57), and ^Mi is the predicted body mass for an individual when the lineal body measurement is standardized to L_0. Weight was measured using a portable XSXScale ES200G x 0.01G (± 0.01g) and forearm length with a Digital vernier Mitutuyo CD-6´´CSX (± 0.01mm).

The second method used to calculate body condition was the hematocrit percentage (%H). Hematocrit is defined as the percentage of the total blood volume occupied by erythrocytes, which depends on the variation in plasma volume, the rate of erythrocyte production and destruction, dehydration, toxins, and direct blood loss, and it may hence be used as an index of the 'health' of the oxygen transport system. The %H was calculated by dividing the total blood draw volume by the erythrocyte volume of each blood sample. This measurement was done with the basal and post stress blood samples (6 h).

Phytohemaglutanin (PHA) challenge

The delayed cutaneous hypersensitivity response was quantified as an indicator of cellular immunity robustness (58–60) in all bats captured. This response was assessed by injecting 50 µL of a PHA solution (3 mg PHA/mL of phosphate buffered saline-PBS) on the right foot and, and 50 µL of PBS on the left foot. PHA influences a variety of cell types and, therefore the response to its injection is complex, but can serve as an index for heightened immune cell activity (61,62). Thickness of the foot was measured before injection and 6, 12 and 24 h

afterwards using digital calipers (Mitutuyo CD-6´´CSX (± 0.01mm). Cellular immune response was estimated as the change in thickness of the PHA-injected foot minus the change in the control foot. Larger localized swelling indicates an increased immune activity. Measurements were made in triplicate and the mean was used for the analyses.

Bactericidal activity (BA)

The antimicrobial capacity of plasma was assessed with the Liebl and Martin Ii protocol (63) as a measurement of serological components (64) (non-specific antibodies (65), complement cascade (66,67), and lysozyme activity (68)). Before assay, the bacteria *Escherichia coli* (ATCC #8739) was reconstructed according to manufacturer instructions. Stock solution of the bacteria was diluted to 1 x 10^5 microbes mL^{-1}. Plasma was diluted (1:23) in sterile PBS, 25 µL of working solutions was added, and samples were incubated for 30 min at 37ºC. After the first incubation, 500 µL of Soy Broth (TSB) were added to samples, which were incubated at 37ºC for additional 12 h. After the second incubation the samples were analyzed spectrophotometrically (Beckman DU-650) at 340 nm. The portion of killed bacteria was calculated as 1- (Sample Abs_{340} / control Abs_{340}). All samples were analyzed in duplicate.

Protein extraction

Erythrocytes samples were first washed twice with 0.9% NaCl by centrifugation at 3000 rpm for 10 min. Protein was extracted afterward by adding lysis buffer (100 µL DTT 1M, 100 µL Phenylmethylsulfonyl flouride (PMSF) 0.1M, 1 complete tablet, 10 ml T-PER) and centrifugated at 13500 rpm at 4ºC for 15 min. Protein samples were separated in four aliquots to prevent frizzing and thawing. Before each assay, total protein concentration was determined spectrophotometrically at 595 nm from each aliquot, using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) (69).

Antioxidant enzyme activity

Antioxidant enzyme activity (AEA) was analyzed spectrophotometrically (Thermo Scientific™ GENESYS 10S UV-Vis; Madison, WI USA) in erythrocytes protein samples as described elsewhere (70). Briefly, Super oxide dismutase (SOD) activity was determined through the xantine/xantine oxidase system, based on protocols by Paoletti *et al* (71). The superoxide anion formed through this system reacts with the nitro blue tetrazolium (NBT) and generates a formazan salt, which was measured spectrophotometrically at 560 nm every 30 seconds for 5 min. One unit of enzymatic activity in this assay is considered as the amount of SOD needed to inhibit 50 % of the superoxide reaction with NBT. Catalasa (CAT) activity was quantified using the protocol established by Abei (72), which evaluates the H_2O_2 decrease at 240 nm every 15 sec for 3 min. One unit of catalase activity (UCAT) was considered as the amount of enzyme necessary to catalyze 1 μmol of H_2O_2 per minute. Glutathione peroxidase (GPx) activity was analyzed at 340 nm with the protocol described by Ahmad *et al* (73). One unit of GPx activity indicates how much enzyme is required to neutralize H_2O_2 using NADPH (74).

Protein oxidative damage

Carbonyls were determined to assess protein oxidative damage by using the DNPH alkaline method (75) and adjusting the optimal volumes for its use in 96 wells plates. Twenty μL of DNPH (10 mM in 0.5 M H_3PO_4) were added to 20 μL of sample protein. Samples were incubated for 10 min in the dark with constant agitation. Afterwards, 10 μL of NaOH (6M) were added and incubated in the dark for 10 min at room at temperature. Absorbance was determined at 450 nm against a blank where the protein solution was substituted by an equal

volume of buffer solution. Carbonyl content was calculated as (Abs_{450}/E)/total protein content of sample, where E = extinction factor of 46.1.

Exogenous GCs and immunization experiment

We tested the effects of a strong stress (by injecting synthetic ACTH) and immune response stimulation (IRS) on antioxidants activity in four experimental groups. Each group was composed of five females captured with mist nets at 21:00 in summer. The first group (ACTH/ PHA), was injected intraperitoneally with 20 μg de ACTH (1.0 ± 0.1 μg ACTH/g body weight) (76) within 5 minutes of capture, and immediately challenged with an subcutaneous injection of 50 μL of a PHA solution (3 mg PHA/mL of phosphate buffered saline-PBS) on the right foot and 50 μL of PBS on the left foot. The second group (ACTH/PBS) was injected intraperitoneally with 20 μg of ACTH, followed by a subcutaneous injection of 50 μL of PBS on both foots. The third group (Saline/PHA) was injected intraperitoneally with 20 μg of saline solution (NaCl 0.9%) followed by a subcutaneous injection of 50 μL of PHA on the right foot and 50 μL of PBS on the left foot. The fourth group (Saline/PBS) was injected intraperitoneally with 20 μg of saline solution (NaCl 0.9%) followed by a subcutaneous injection of 50 μL of PBS. After treatments, bats were kept individually in small cotton bags during 42 h, they were checked every 2 hours for fecal samples. Feces were collected and maintained in liquid nitrogen for analyses of cortisol. A blood sample (300 μL) was collected 24 h after the treatment and maintained in liquid nitrogen, until its shipment to the laboratory in dry ice, were it was stored at -80ºC for analysis of antioxidant activity and carbonyl concentration. Bats were fed twice a day during their captive period, and the food was dyed with blue vegetal pastry dye. Bats were allowed to eat freely, and the amount of pieces they ate was recorded. However, as not all bats ate the same amount, and the number of defecations of each bat was not equal, so paired analysis of ANOVA was not possible. ITT was 10.7 ± 3.6

h (mean ± SD n= 17) so FCM samples were grouped as basal samples (ITT: 3.1 ± 2.8 h) collected up to 5 h of capture; 2nd samples collected within 6 h >12 h, and 3rd samples within 12 ≥ 24, and 4th samples collected > 24 h.

Weather data

Data for T_a and wind speed (W) during the days we had collected the samples were obtained from the nearest meteorological station in Bahía de los Ángeles, Baja California, Mexico. Data was recorded at the station every 10 seconds. We used the daily median ambient temperature ($dŦ_a$) and the median night wind speed ($\hat{W}_n$) for the analyses. Considering that bats are mostly affected by wind during their foraging hours, we used the data recorded between 19:00 and 23:59 hours to obtain the median night wind speed ($\hat{W}_n$) of the sampled days.

Statistics

We tested seasonal changes in fecal cortisol metabolites (FCM) using 1-way ANOVA for log-transformed values for the basal and SR1 groups, and a Kruskal-Wallis analysis for the SR2 group. Post-doc Tukey and Dunn's tests were respectively performed when necessary. To test whether the effect of movement restriction on FCM concentrations, we performed a paired t-test in all comparison groups except for the comparison between the SR1 and the SR2 group in early winter, where we performed a Wilcoxon paired test. To evaluate whether FCM influenced antioxidant activity, oxidative damage and immunocompetence, we performed linear regressions for each variable. A 2-way ANOVA test was performed to compare the four treatments challenged with ACTH and IRS, with the 4 sample set points (Basal, 2nd, 3rd,and 4th). Tukey post hocs were performed to compare differences between treatments within each time set point, changes in FCM in each treatment over time, and differences in total FCM

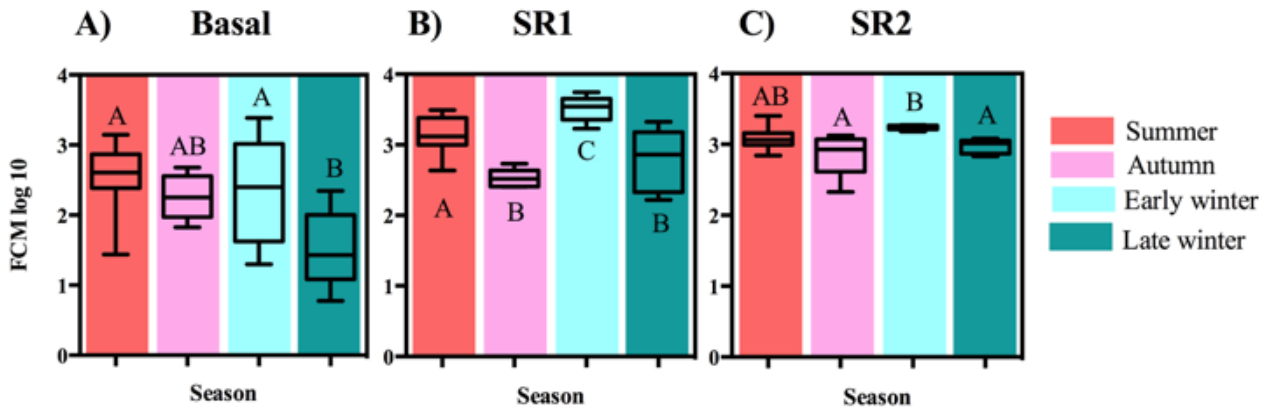
excreted over the 48 h experiment between each treatment. Finally, we performed a 1-way ANOVA- with Tukey post-hoc tests to determine the effect of each ACTH/immunization treatments in antioxidant activity and inflammation response.

Results

Seasonal changes

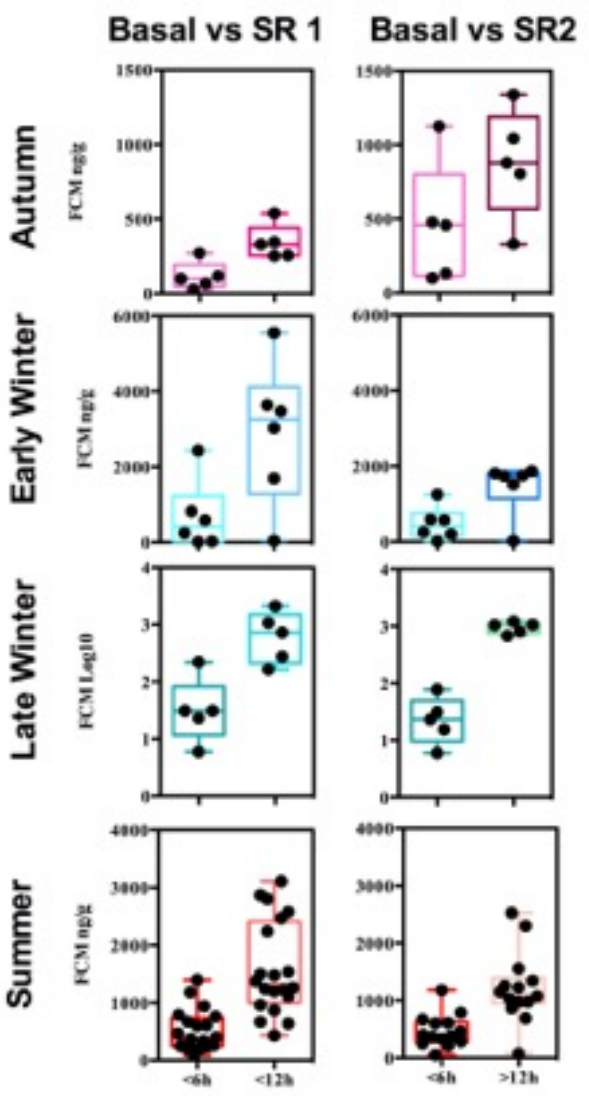
There were significant seasonal differences ($F_{3, 36}$ = 5.78, P = 0.0025) in FCM of basal samples, between those of late winter compared to summer (P = 0.0011) and early winter (P = 0.0221; Fig 1). FCM samples from SR1 were significantly different among seasons ($F_{3.31}$ = 15.05, P < 0.0001). Samples collected in early winter had higher FCM concentrations than those collected from summer (P = 0.0352), autumn (P < 0.0001), and late winter (P = 0.0005), and FCM concentration was higher in summer than in autumn (P = 0.0002) and late winter (P = 0.0376; Fig 1). FCM samples from SR2 varied seasonally ($H_{3.27}$ = 11.14, P = 0.0110) with higher values in early winter compared to autumn (P = 0.0210) and late winter (P = 0.0301) (Fig 1).

Figure 1. Fecal cortisol metabolites (FCM) of bats by season, on the different group time samplings. Box and whiskers graph with mean, Min-Max values, seasons are indicated by colored bars. A) Baseline samples, collected 0-5 h after capture; B) Stress reactivity 1 (SR1) samples collected 6-14 hours after capture; C) Stress reactivity 2 (SR2) samples collected 15-24 hours after capture. ANOVA analysis of FCM compared between seasons, Different literals mean significant differences (P < 0.05).



We found lower FCM concentration in all seasons in basal samples compared to SR1 (autumn: $t_4 = 18.73$, $P < 0.0001$; early winter: $t_5 = 4.04$, $P = 0.0099$; late winter: $t_5 = 5.06$, $P = 0.0072$; summer: $t_{19} = 6.26$, $P < 0.0001$; Fig. 2) and SR2 (autumn: $t_4 = 3.28$, $P = 0.0304$; early winter: $W_4 = 21.00$, $P = 0.0313$; late winter: $W_4 = 7.49$, $P = 0.001$; summer: $W_{13} = 4.46$, $P = 0.0006$; Fig 2).

Figure 2. Fecal cortisol metabolites (FCM) change after acute stress by movement restriction. Box and whiskers graph with mean, Min-Max values. Left column: comparison between FCM basal samples (collected 0-5 h after capture) and stress reactivity 1 (SR1) samples (collected 6-14 h after capture). Right column: comparison between FCM basal samples and stress reactivity 2 (SR2) (collected 15-24 h after capture). A paired t-student was performed in each case. All comparisons were significant ($P < 0.05$)



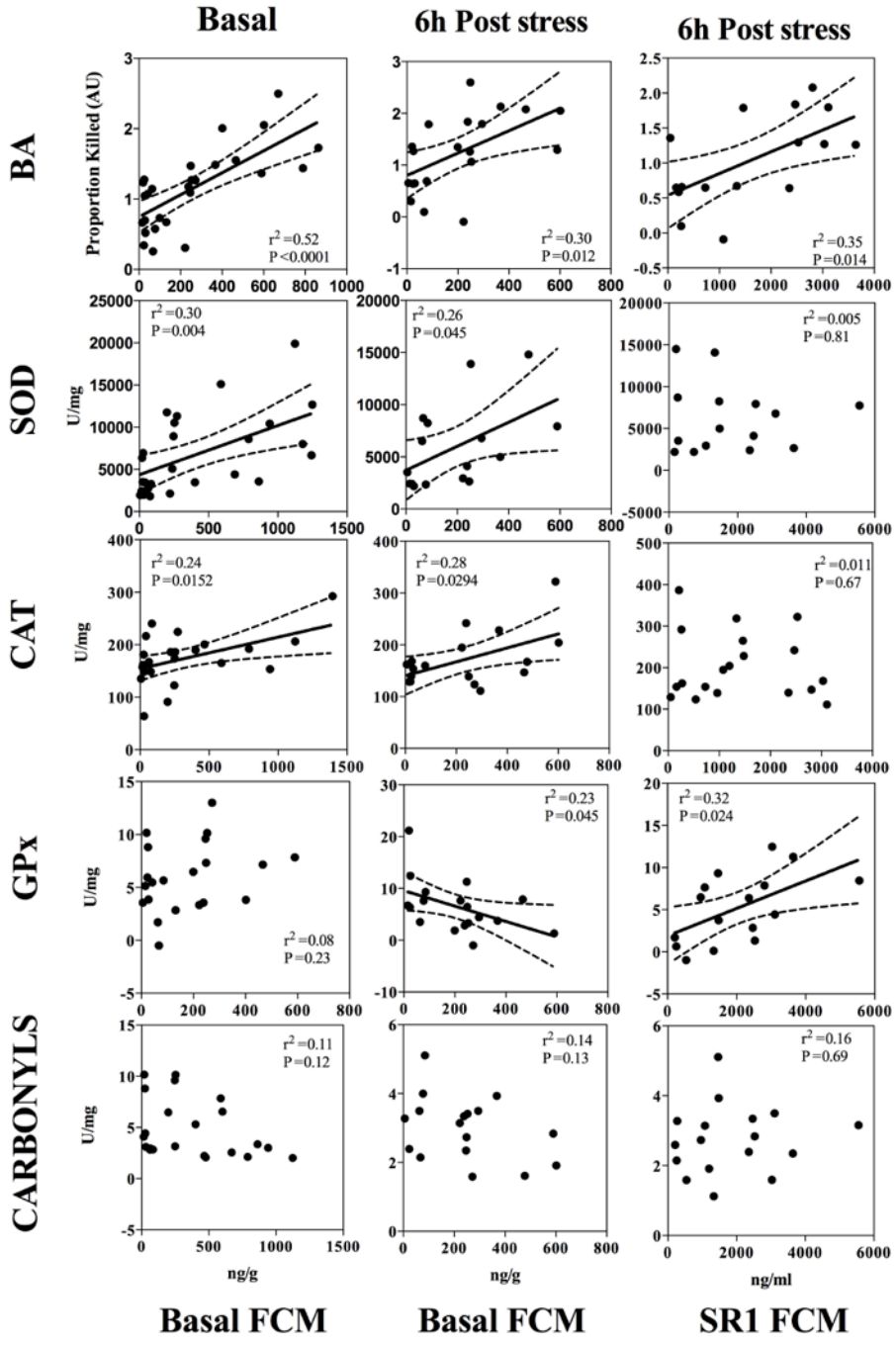
Relationship with other physiological parameters

Basal FCM was positively correlated with basal levels of: BA ($r^2 = 0.52$, $P < 0.0001$; Fig. 3), SOD ($r^2 = 0.30$, $P = 0.0040$; Fig. 3), CAT ($r^2 = 0.24$, $P = 0.0152$; Fig. 3), SMI ($r^2 = 0.22$, $P = 0.0188$; Fig. 4) and H% ($r^2 = 0.23$, $P = 0.0108$; Fig. 5). In contrast, there was a negative relationship between basal FCM and the PHA index at 24 h ($r^2 = 0.23$, $P = 0.0125$; Fig. 4). In addition, basal FCM was positively correlated with $d\bar{T}_a$ ($r^2 = 0.34$, $P = 0.0004$; Fig. 5), and negatively correlated with $\hat{W}_n$ ($r^2 = 0.34$, $P = 0.0004$; Fig. 5). Furthermore, basal FCM was positively correlated with post 6 h of stress values of: BA ($r^2 = 0.30$, $P = 0.0121$; Fig. 3), SOD ($r^2 = 0.29$, $P = 0.0369$; Fig. 3) and CAT ($r^2 = 0.28$, $P = 0.0338$; Fig. 3) activities and negatively

correlated with GPx activity ($r^2 = 0.22$, P = 0.0479; Fig. 3) measured after 6 h of acute stress. There was no significant correlation between basal FCM and protein damage ($r^2 = 0.14$, P = 0.1330; Fig. 3).

FCM values from SR1 were positively correlated with BA ($r^2 = 0.36$, P = 0.0142; Fig. 3) and GPx ($r^2 = 0.32$, P = 0.0236; Fig. 3) activity after 6 h of stress and PHA index after 12 h of stress ($r^2 = 0.18$, P = 0.0291; Fig. 4). These FCM values were not significantly related to SOD ($r^2 = 0.005$, P = 0.8084; Fig. 3) and CAT ($r^2 = 0.01$, P = 0.6661; Fig. 3) activities after 6 h of acute stress, protein damage ($r^2 = 0.01$, P = 0.6987; Fig. 3), SMI ($r^2 = 0.09$, P = 0.1381; Fig. 4), H% ($r^2 = 0.06$, P = 0.3812; Fig. 5), PHA index at 24h ($r^2 = 0.38$, P = 0.4266; Fig 4), $d\text{Ŧ}_a$ ($r^2 = 0.0007$, P = 0.8807; Fig. 5), and $\hat{W}_n$ ($r^2 = 0.04$, P = 0.2952; Fig. 5).

Figure 3. Linear regression between fecal cortisol metabolites (FCM) and oxidative stress and immunocompetence markers. Top headings represent time samples of antioxidant activity (SOD, CAT and GPx), Bactericidal activity (BA) and carbonyls concentration. Bottom headings represent Time samples of FCM: basal samples (collected 0-5 h after capture) and stress reactivity 1 (SR1) samples (collected 6-14 h after capture). Significant regressions ($P < 0.05$) are plotted with regression line (full line) and error bars (side-dashed lines). Non-significant regressions ($P > 0.05$) were plotted without line or error bars.


Basal
6h Post stress
6h Post stress
BA
SOD
CAT
GPx
CARBONYLS
Proportion Killed (AU)
U/mg
ng/g
ng/ml
r2 =0.52
P<0.0001
r2 =0.30
P=0.012
r2 =0.35
P=0.014
r2 =0.30
P=0.004
r2 =0.26
P=0.045
r2 =0.005
P=0.81
r2 =0.24
P=0.0152
r2 =0.28
P=0.0294
r2 =0.011
P=0.67
r2 =0.08
P=0.23
r2 =0.23
P=0.045
r2 =0.32
P=0.024
r2 =0.11
P=0.12
r2 =0.14
P=0.13
r2 =0.16
P=0.69
Basal FCM
Basal FCM
SR1 FCM

Figurer 4- Linear regression between fecal cortisol metabolites (FCM) and inflammatory response (PHA challenge) and Scaled Mass Index (SMI). Bottom headings represent Time samples of FCM: basal samples (collected 0-5 h after capture) and stress reactivity 1 (SR1) samples (collected 6-14 h after capture). Significant regressions ($P < 0.05$) are plotted with regression line (full line) and error bars (side-dashed lines). Non-significant regressions ($P > 0.05$) were plotted without line or error bars.

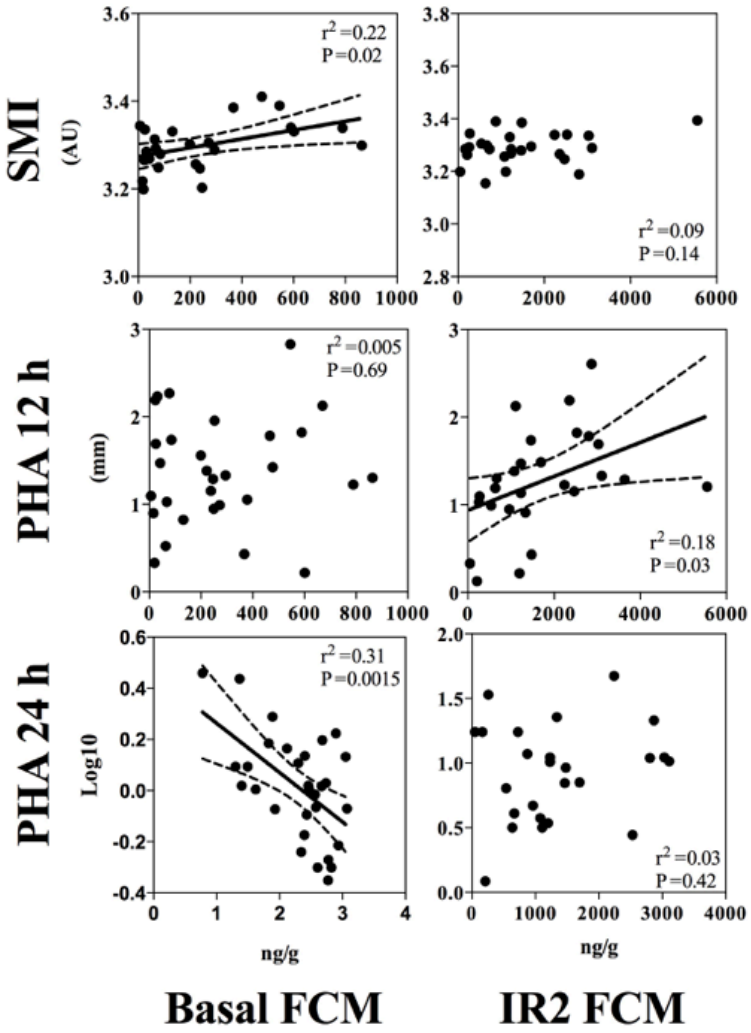
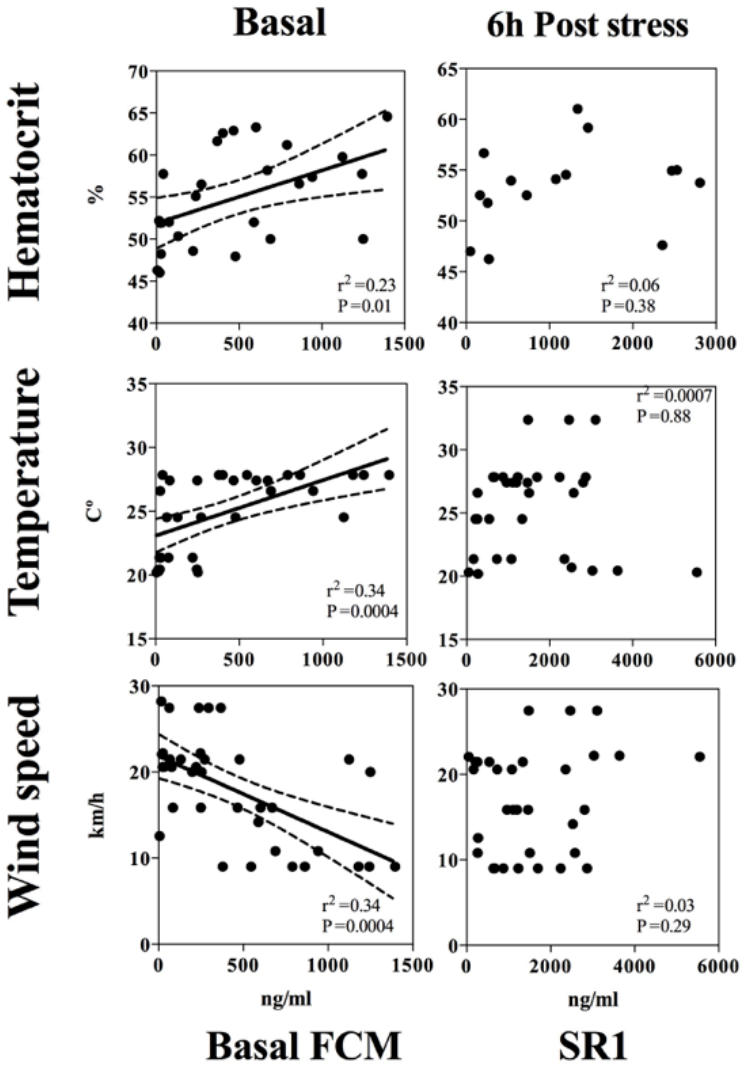


Figure 5.- Linear regression between fecal cortisol metabolites (FCM) and Hematocrit percentage, daily median ambient temperature and median night wind speed. Top headings represent time samples of hematocrit %, wind speed and temperature. Bottom headings represent Time samples of FCM: basal samples (collected 0-5 h after capture) and stress reactivity 1 (SR1) samples (collected 6-14 h after capture). Significant regressions ($P < 0.05$) are plotted with regression line (full line) and error bars (side-dashed lines). Non-significant regressions ($P > 0.05$) were plotted without line or error bars.



ACTH and Immune response stimulation (IRS)

Fecal cortisol metabolites (FCM) concentration did not change significantly over time after Saline/PHA and ACTH/PHA treatments, but it did with ACTH/PBS and Saline/PBS treatments ($F_{3,55}$ =3.29, P =0.0271), which had two peaks in FCM at 2nd and at 4th sample time points with higher FCM concentrations than basal and 3rd sample points (P s < 0.05) (Fig. 6A).

There were significant differences when comparing FCM concentrations between treatments among time groups ($F_{3, 55}$ =7.63, P =0.0002). There were no significant differences in basal FCM between treatment groups. However, in the 2nd time group the ACTH/PBS treatment had significantly higher FCM concentrations than the Saline/PBS (P =0.0211), Saline/PHA (P =0.0005) and ACTH/PHA (P =0.0152) treatments. In the FCM samples from the 3rd time point, there were no significant differences between treatment groups in FCM values from samples collected a. Finally, for the FCM samples for the 4th group, treatments were different; ACTH/PBS increased once again and was higher than Saline/PHA (P =0.0001), Saline/PBS (P =0.0079) and ACTH/PHA (P =0.0001) (Fig. 6B).

The total GCs excreted in the 42 h of the experiment was significantly different between groups ($F_{3,55}$ =2.97, P =0.0059); the ACTH+PBS group had the highest total FCM concentrations compared to Saline/PBS (P <0.0001), Saline/PHA (P =0.0003), and ACHT/ PHA(P =0.0036) (Fig. 6C).

Inflammation after IRS varied with treatment ($F_{3.16}$ =33.26, P <0.0001); after 12 h, it was the same in ACTH/PHA, and Saline/PHA treatments (P =0.8917); but it was higher than both treatments without PHA (P s <0.05) (Fig. 7). Inflammation after 24 h post IRS also varies among treatments ($F_{3.16}$ =32.27, P <0.0001): it was higher in the ACTH/PHA and Saline/PHA treatment compared to the ACTH/PBS (P =0.0030; P <0.0001 respectively) and Saline/PBS (P <0.0001 respectively) treatments. But ACTH/PHA was lower compared to the Saline/PHA

treatment (P =0.0199) (Fig. 7). CAT activity varied among treatments ($F_{3,8.2}$ =33.87, P <0.0001): the activity was lower in the Saline/PBS treatments than in ACTH/PBS (P =0.0026), and Saline/PHA treatments (P <0.0001), it was not different than in the ACTH/PHA treatment (P =0.1245). No other treatment comparisons (ACTH/PBS vs ACTH/ PHA: P =0.9657 ACTH/ PBS vs Saline/PHA: P =0.6586; ACTH/PHA vs Saline/PHA: P =0.9996; Fig. 7) were significantly different after 24 h. GPx activity varied significantly among treatments ($F_{3,16}$ =7.68, P = 0.0021); activity was higher for the Saline/PHA treatment than the Saline/PBS (P =0.0047), ACTH/PBS (P =0.0061), and ACTH/PHA treatments (P =0.0087; Fig. 7). SOD activity varied among treatments ($F_{3.16}$ =6.48, P = 0.0044); the activity in the ACTH/PHA treatment was lower than in the Saline/PBS (P =0.0035) and ACTH/PBS treatments (P =0.0262), but it was no significantly different than in the Saline/PHA treatments (P =0.2152; Fig. 7).

Figure 6. - Fecal cortisol metabolites change for the ACTH/Immunization experiment.

A) FCM change over time after treatments injection (mean ± SD), capital letters means significant differences ($P < 0.05$), Treatments identified by colors. B) FCM comparison of treatments by sampling times groups; capital letters means significant differences ($P < 0.05$) within each time group. C) Compression of total FCM excreted during experiment between treatment groups, capital letters means significant differences ($P < 0.05$).

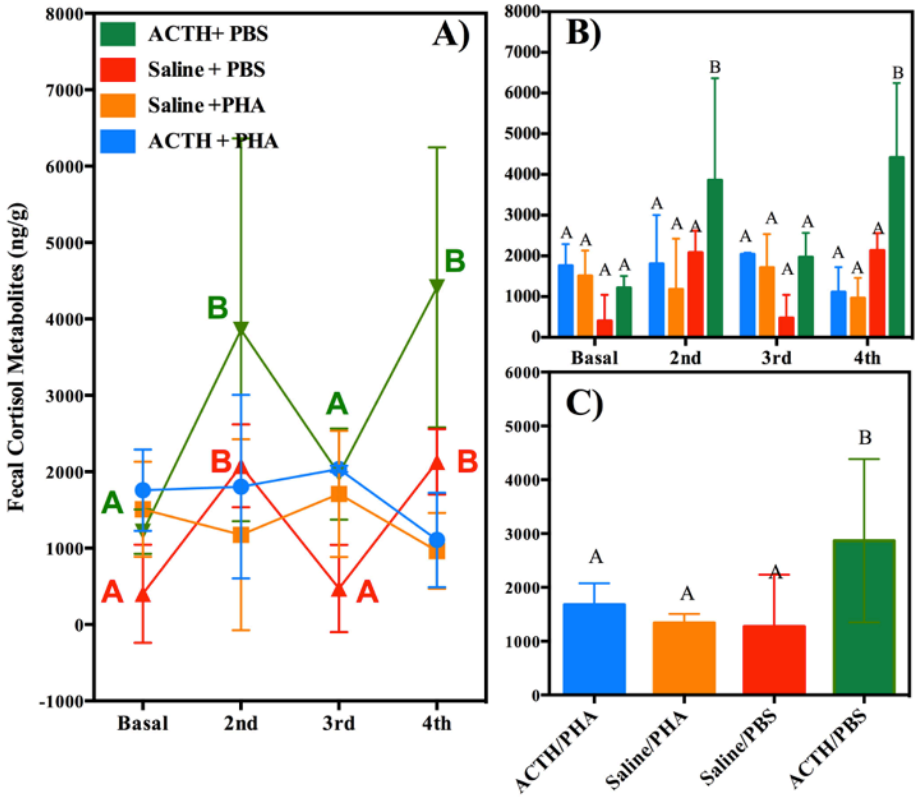
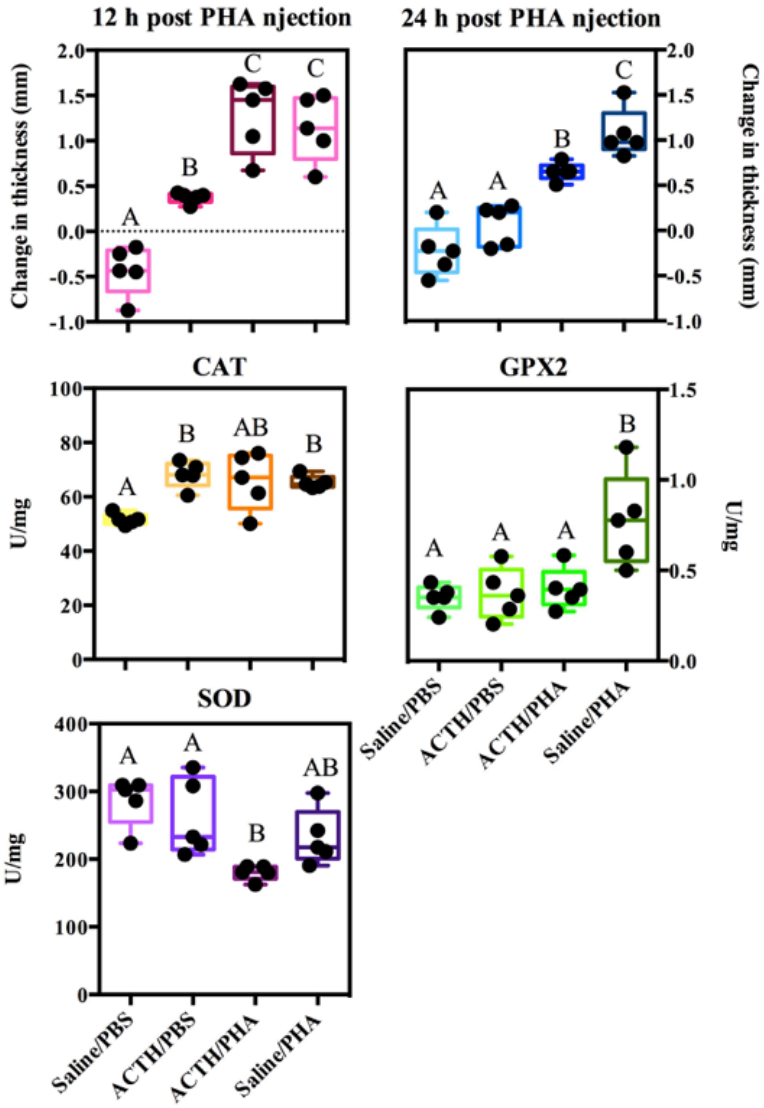


Fig 7. Effect of ACTH/Immunization treatments on inflammatory response and antioxidant activity.

Capital letters means significant differences between treatments ($P < 0.05$).



Discussion

Our objectives were to determine the change on basal seasonal FCM thorough the year to determine how *M. vivesi* responded to its environment through their life history. After which we tasted how a stress stimulus affected their FCM concentrations depending the season of the year. At the same time we tested the relationship between FCM-immunocompetence-redox state, for which we expected to find a positive relationship between FCM and immunocompetence under short-term stress and a negative relationship with prolonged stress. Like wise we expected antioxidants to be positively related to GC concentrations with no oxidative stress generated.

Seasonal changes

Significant effects of season or weather conditions such as temperature, humidity, and availability of food and water on fecal GCM concentrations have been shown for several species of mammals and birds, with most studies reporting higher levels during harsher conditions in winter or during the dry season (reviewed in (77)). Other seasonal activities, such as breeding, lactation and hibernation are also known to influence basal GCs levels in a wide range of species. In contrast to our expectations, we found little variation in FCM levels of *M. vivesi* through the year. Basal FCM samples displayed similar levels during summer, autumn and early winter, while concentrations in late winter were lower. Although, it has been reported that GCs tend to peak during the breeding season in a wide range of free-living vertebrates, (16), this might not necessarily be for all species, and It might vary depending of the specific stage of breeding . GCs were no different for hamsters lactating (*Cricetus cricetus*) in comparison to non-reproductive individuals (78), although baseline GCs levels were higher during late pregnancy for flying fox (*Pteropus hypomelanus*) relative to early

pregnancy and lactation (40). In our study, gestating females were not sampled; therefore this could also partially explain why bats had similar basal FCM during autumn (non-breeding season), summer (lactation period) and early winter (mating period). Milk production is an important physiological pathway for nutrients and glucocorticoid signaling that potentially influences offspring growth and behavioral phenotype (79). High GCs during lactation might be a signal to offspring's of a harsh environment, shaping their phenotype to a lower reactivity to stress (80). This data suggest that fish eating bats maintain similar baseline GCs from summer compared to autumn and early winter as a signal of their adaptation to their environment. In addition to lactation, summer has the higher temperatures of the year (81), and it has been reported that mammals living in naturally hot climates maintain low GCs levels as an adjustment to maintain thermal balance (82).

High GCs levels in plasma, have been reported for the little brown bat (*Myotis lucifugus*) during the beginning of the hibernating season after which they progressively decreased by the end of this season (from 1.13 ± 0.16 μg/ml in November to 0.76 ± 0.09 μg/ml in March) (41). GC´s play an important role during torpor (42,43). They increase availability of circulating glucose and triglycerides by stimulating gluconeogenesis from amino acids in the liver, by inhibiting glucose uptake from tissues other than brain and, especially relevant to hibernation, by encouraging lipolysis (43). High GCs have been reported during pre-hibernation to encourage fattening, and at the beginning and midst of hibernation to preserve glucose in the circulation to fuel metabolism in the brain during torpor and active metabolism (41,43,83). Therefore, this could partially explain why FCM was higher during early winter, than at the end of the hibernation season in late winter. Furthermore, the low FCM during late winter could mean that the ambient conditions were not stressful, as suggested by the low antioxidant activity and low oxidative stress during this period [68], and the warmer ambient temperatures during late (21.2ºC, 16.7±25.4ºC) than early winter (20.0º C, 16.3±24.4ºC) (median±SD).

However, we found that when assessing bats reactivity to an acute stress stimulus, FCM concentration differed between seasons. FCM concentration at SR1 were higher in summer and early winter, than in bats during autumn and late winter. Thus, although their basal FCM levels were not different between autumn, early winter and summer, the response to stress was stronger during the hot/lactation period, and during the beginning of hibernations/cold season. Furthermore, stress response related to antioxidant activity and bactericidal capacity of *M. vivesi* is also similar between summer and early winter (81). However, when bats were exposed to longer stress period (SR2), FCM in autumn and late winter were similar to summer, but in early winter FCM levels were still the highest. This could suggest that bats during the beginning of winter are more susceptible to stress than any other season, probably due to the combined effect of the colder weather (compared to the other seasons), together with low body condition (which could suggest reduced foraging) (81)

Therefore, although no seasonal drastic differences were found for basal FCM concentrations, seasons do influence the intensity of the stress response, at least for short-lasted stress stimuli.

Relationship with other physiological parameters

Most of the correlations found in this study had low determination coefficients ($r^2 < 50\%$). However, high r^2 are difficult to obtain in natural populations, since animals are exposed to a number of different variables, which we cannot control unlike in laboratory settings. Also, it is known that there is a considerably intrinsic variability in GCs levels between individuals from the same population due to differences in personalities (84,85), which are also difficult to identify in the field. Nonetheless, our results are still good indicators of the relationships between the physiological parameters in the population. Bats with high SMI scores and high

hematocrit levels tended to have high basal FCMs, but their response to acute stress was independent of their body condition. Bats with high basal FCM also had high basal bactericidal capacity, SOD and CAT antioxidant activity, but low inflammation response (PHA index at 24 h). Bats with high basal FCM had high bactericidal capacity and high antioxidant (SOD, CAT and GPx) stress responses after 12 h of movement restraint, which suggest that animals that have high basal glucocorticoids levels have a strong antioxidant and humoral response to stress. However, the levels of FCM after a stress stimulus (SR1) was positively related only to stress levels of BA and GPx, which suggest that FCM stress level are not necessarily related to all the antioxidant activity. Furthermore bats increased their SOD and GPx activity during summer and early winter after a 6 h acute stress (81), and during these seasons they also had high SR1 and SR2 FCM. Also, SR1 FCM was higher for early winter compared to summer, but bats only experienced oxidative damage after longer acute movement restriction (12 h), during summer (81). Therefore, our results contrast with reports in adult male Wistar rats that indicated that GCs impairs antioxidant activity (86) It also contradicts reports of GCs causing oxidative damage (36,87,88), although most of these studies focus on the chronic exposition to GCs, rather than in acute expositions. Therefore, our study suggests that under short periods of time, high GCs response would be beneficial and might positively influence the prospects of survival.

On the other hand, relationship between GCs and immune response seems to be more complex. PHA-induced swelling is used as an index of T cell-mediated immunocompetence, but other leukocytes (basophils, neutrophils, and eosinophils) are also responsible for localized vasodilatation, infiltration and edema(30,65,66). Additionally, the number of lymphocytes present before PHA injection- positively affects the number of lymphocytes infiltrating into the tissue (89), suggesting that the levels of basal leukocyte populations determines the intensity of the response when it is presented with a novel antigen. At 12-24 h post injection,

neutrophils, lymphocytes, and macrophages are at their peak (13,30,31,90). Therefore, our findings further support the anti-inflammatory role of glucocorticoids, as bats with higher basal FCM had lower inflammation. However, this relationships shift to a positive one under short-stress conditions, as shown by the positive correlation between inflammation and the FCM levels after 12 h of movement restriction. This supports the hypothesis that short-stress enhances immunocompetence (30), which as far as we know, is demonstrated for the first time, for a free ranging animal. Furthermore, FCM levels were always positively correlated to BA, suggesting that in the short term, GCs enhance related humoral activity. Bactericidal activity (BA) primarily measures the complement cascade and the lysozyme activity (91), and it has being previously reported that GCs increase in peripheral monocytes (92), the expression of complement 3 of the cascade (C3). Similar effects were reported in studies of lung epithelial cells and hepatocytes (93). Glucocorticoids may therefore have a counter-repressive action on local complement functions (93).

ACTH and Immunization challenge

Immune response stimulation (IRS) at the moment of stress exposure had a dampening effect on the stress response of animals. All groups of bats had similar basal FCM, however bats treated with ACTH/PHA had significantly lower FCM excretion than stressed non-IRS bats (ACTH/PBS). Both groups of bats without the IRS (Saline/PBS and the ACTH/PBS treatments), had the exact same pattern of change over time, with peaks of FCM levels at the 2nd and at 4th sample time, only differing in the intensity of such response. However, IRS groups of bats (Saline/PHA and ACTH/PHA treatments) had no change on FCM concentration over time, and the group injected with exogenous ACTH had lower stress response. There could be two possible explanations for this; one is that in the face of an immune challenge, organisms react by lowering their GC response in order to cope with the immune threat.

Similar reports had been found in a tropical population of House Sparrows (*Passer domesticus*) that showed low basal and stress GC levels; Furthermore, their response to PHA injection was not affected by GC implants, but in a temperate population with higher basal and stress GC levels the immune response was suppressed [86]. Also, a study in air-breathing fish (*Anabas testudineus*) demonstrated that an immune challenge suppresses the cortisol-driven stress response (94). Therefore, our results and reported literature, suggest that animals which encounter an immune challenge may prioritize immune response over stress response, or that animals that live in areas where disease threats are high, might maintain low baseline and stress-induced levels of GC (95). Alternatively, PHA injection might enhance GC activity; for example, stimulation with PHA increases GC receptors activity by 3-fold in lymphocytes (96), those GC are bound to a target and therefore excretion of the hormone could be delayed. Accordingly, our results show that the inflammation produced by the PHA injection was the same in bats treated with ACTH/PHA or Saline/PHA after 12 h of restriction, moreover the inflammation in the Saline/PHA group was greater compared to the ACTH/PHA group after 24 h. This result corroborates the anti-inflammatory effect of GCs, even when FCM concentrations were the same in both groups. This suggests that bats on the ACTH/PHA treatment had more GCs bound to their target receptor and that they are not being excreted. Quantification of free GC in plasma in the treatment groups would help to examine this possibility.

Moreover, bats on the Saline/PHA treatments were subject to increased amounts of endogenous GCs produced by movement restriction, but their concentration was lower than the stress produced by exogenous GCs stimulated by ACTH injection Therefore, we propose that under a mild stress situation, acute stress can enhance cell-mediated immunity, but this is inhibited after a certain threshold of stress intensity, even if stress is short.

The activity of each antioxidant enzyme was affected in different ways by the treatments. CAT activity was equally enhanced by the strong stress treatment (ACTH/PBS) and by the immunization treatment (Saline/PHA), but not when bats were exposed to stress and immunization simultaneously (ACTH/PHA treatment). GPx activity was not enhanced by stress but it was by immunization (Saline/PHA), which suggest that GPx scavenger activity is strongly related to the immune oxygen burst, rather than to the stress response, as also reported by (81,97). SOD activity remained the same in almost all groups, but its activity dropped in bats exposed to stress and an immune challenge (ACTH/PHA). Our results demonstrate that each enzymatic antioxidant reacts differently to stress and immune challenge, and that, their activity is compromised when the animals confront both a stressful situation and an immune challenge at the same time.

Our findings supports the idea that animals lower their basal GC levels when living in challenging environments, but their response to acute stress differs seasonally. However, just as with antioxidant activity, GCs response to stress do not vary through the year when bats are exposed to longer periods of stress. It seems, that bats had a positive relationship between antioxidant activity and glucocorticoids, so more studies on the short term effects of GCs on different physiological parameters are measured in order to further understand its implication in fitness, survival and evolution and to make them available as good markers of animal's welfare.

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Redox state changes in several tissues from *Myotis velifer* after hibernation interruption

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Introduction

The inability of non-hibernating animals to survive prolonged hypothermia assaults and metabolic depression, suggests that hibernators must count with physiological strategies to overcome the risks of damage during hibernation and arousal in order to ensure survival (1). Individuals may, therefore, be exposed to relevant physiological costs, mainly expressed in terms of oxidative damage and energy consumption needed to keep the antioxidant defenses up-regulated, and to activate the repair systems. It is possible that the need to regulate the redox system exposes individuals to a number of trade-offs and, therefore, oxidative stress could have represented a relevant modulator of life-history strategies (2). Moreover mammalian hibernators are a unique natural model to study resistance to the deleterious effects of low tissue perfusion and reperfusion (3).

When animals enter torpor, heart rate and cardiac output quickly decrease to 50%, while at the same time; body temperature (T_b) only decreases 1ºC. This could result on the perfusion of organs that are still performing biochemical reactions at euthermic rates, since oxygen concentration declines from normoxia to hypoxia (3–5). Then, cellular hypoxia occurs, and at some point during that phase, mitochondrial reactive oxygen species (ROS) formation increase temporarily (5). In the brain, which requires one-fifth of the total oxygen consumed (6,7), there is a reduction in O_2 consumption by as much as 50-fold and a 10-fold in cerebral blood flow (6). Furthermore, the respiratory rate is reduced from 100-200 to 4-6 breaths/min, subjecting the organism to prolonged periods of apnea, which could be especially stressful for the lungs (3).

During arousal, there is a fast increase in heart rate, ventilation and metabolism before T_b has been completely reestablished, which potentially increase the risk of oxidative damage (8).

Since mitochondrial and NADPH oxidases increase ROS production, antioxidant defenses might become outrun (3,5,6).

Reperfusion as a physiological process in animals adapted to these challenges – anoxia/ hypoxia or freezing – is an integral part of their natural life cycle (7). It has been demonstrated that ischemia-reperfusion insults do not produce damage in these kind of animals, when compared to non-hibernating animals (6). There have been reports on an increase in antioxidant defenses during anoxia/hypoxia or estivation in different organisms (9–12). However, the mechanism by which torpid mammals adapt to oxidative stress during rewarming remains unresolved. It has been proposed that certain defensive pathways against oxidative stress are activated during arousal from torpor (13). Or that a more severe insult is required to produce a pathologically equivalent response in euthermic hibernators animals compared to hibernating animals because of enhanced antioxidant defense during hibernation (14). Therefore, hibernators seem to deal with potential oxidative damage by enhancing antioxidant defenses in an anticipatory manner (15). However, the majority of studies on oxidative stress of hibernating mammals are focused on the difference between torpid animals and active animals (16–22). Yet, winter torpor or hibernation, typically consists of numerous discrete torpor bouts separated by brief periods of normothermy, or arousal, which are proposed to serve numerous physiological purposes (23–26). But only a handful of studies have focused on the changes that occur at the moment of arousal, when the oxidative insult takes place (7,14,27–30); and most of these studies have focused only in analyzing a single, or few, tissues or antioxidants, when findings until now have made it clear that results might vary depending on the tissue being tested.

Oxidative insult occurs on a short time scale during arousal. There are numerous reports which states that oxygen consumption peaks when body temperature reaches near euthermic ranges. In Syrian hamsters (*Mesocricetus auratus*) there is a peak in ROS generation, measured as uric

acid concentration when body temperature reaches 32ºC. Also the respiratory rates steadily increased and went up to above normal euthermic levels in mid to late arousal (peak at 25–32 °C) (14). In bats, *Rhinolopus ferrumequinum*, V_{O2} peak occurred 30 min after arousal (8.7-fold higher than their resting metabolic rate), when their T_b was 34.4 ± 2.4ºC (30)

Therefore the aim of this work was to determine the changes in redox state in the cave myotis bat, *Myotis velifer* by measuring antioxidants activity (SOD, CAT, and GPx), along with protein damage in a wide range of tissues; blood, brain, heart, lungs, liver and white adipose tissue (WAT), in torpid and in aroused bats, 30 minutes after interrupting their hibernation state, when bats had reached 30ºC of body temperature.

Materials and methods

Chemicals

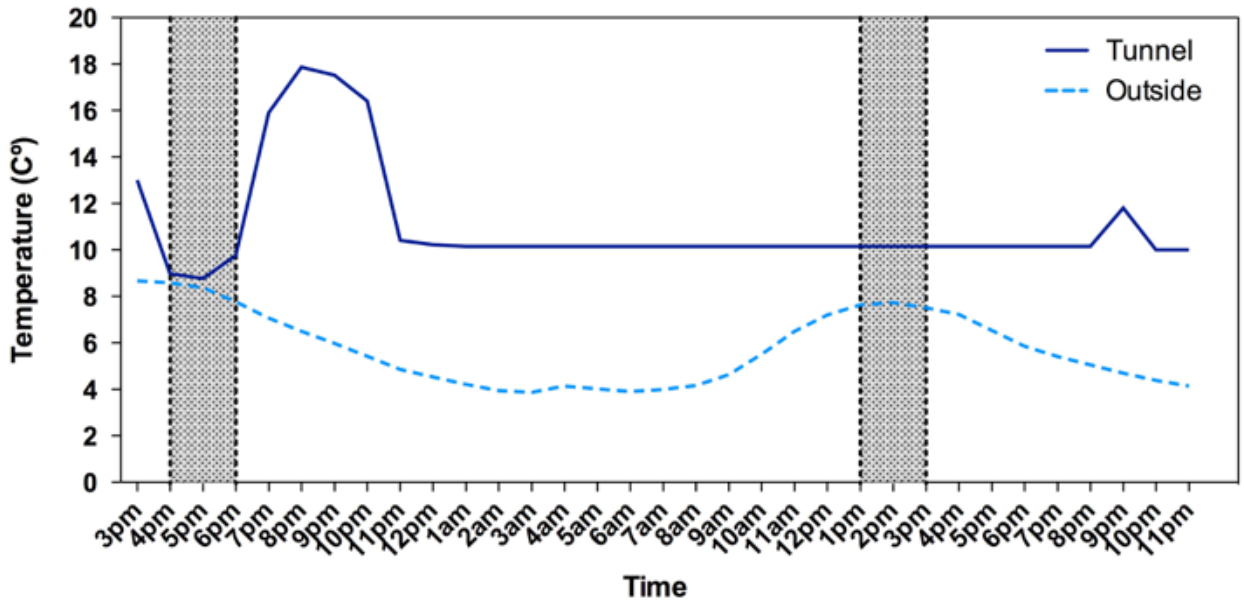
All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The reagents obtained from other sources are detailed throughout the text.

Study site, animal and tissue acquisition

M. velifer bats were captured in a tunnel located between the municipalities of Puebla and Tlaxcala (19°37'14"N, 98°02'02"W; 3,220 m.a.s.l.). The tunnel is collapsed at the middle, the side located in Tlaxcala is flooded, and water is intubated for the community use. But locals don't enter through the tunnel.

We entered the tunnel through the day with neoprene suits. Material and bats cages were transported in an inflatable boat. In the deepest part, water reached chest length at the entrance of the tunnel, but as we advanced we reached a dry zone. Ambient temperature was registered with HOBO temperature data loggers, one outside the tunnel (5.76 ± 1.58 ºC) and one inside the tunnel (11.03 ± 2.34) mean ± SD (Fig. A).

Figure A. Graphic representation of ambient temperature outside the tunnel and inside the tunnel over a 32 h period.



Hibernating *M. velifer* individual bats were identified, as being in torpor state when individuals were covered in a thin layer of ice, and respiratory rates were indistinguishable by sight. *M. velifer* skin temperature tends to fall within the range of that of the ambient temperature (31), and its minimum and maximum oxygen consumption in ml gl^{-1} hr^{-1} at 10ºC is of 0.1 and 8.8, being highest at the first 24 h of exposure to low temperature, after which it reminds constantly low (31). *M. velifer* mean body temperature at thermoneutrality is reported to be of 36.0-36.2ºC (32). Individuals, with body temperature bellow 12 ºC were considerate to be in deep torpor, as previous studies in this species report (32,33). Skin temperature (T_{skin}) (34–36) and fur temperature (T_{fur}) were taken as a proxys to body temperature, measured with an infrared thermometer (General Tools IRT855DL 50:1) by triplicate (IR-T_{fur}). Bats in the 'Torpor group' had a mean IR-T_{fur} of 7.57 0.46 ºC and the 'Arousal group' of 7.49 0.57 ºC (table 1). Bats were taken with care and quickly measured a second time with a thermocouple connected to the k-port of the IR thermometer by triplicate (TIR-T_{skin}), by placing it inside the

ear. A group of five bats was sacrificed immediately (Torpor group) by decapitation. Their TIR-T_{skin} averaged 11.01 0.84 ºC at the moment of death. Blood was collected with heparinized tubes and stored in ice. Bats were then put on ice and taken out of the tunnel. Organs were dissected immediately after, in a near cottage set as temporary lab. A second group of five bats (Arousal group) was gently aroused after the second TIR-T_{skin} (average 9.89 0.74 ºC) measurement, and put into a cage to allow full arousal. That group was sacrificed after 30 min, when bats were actively moving around the cage; mean TIR-T_{skin} was of 30.74 1.56 ºC at the moment of death.

All organs were divided in two and placed in eppendorff tubes. Blood was centrifuged at 3000rmp and plasma and red blood cells (RBD) were separated. All samples were stored in dry ice until their translation to the laboratory.

All bats were euthanized by decapitation according to AVMA regulations (37), and permit of SEMARNAT FAUT-0159.

Protein extraction

Blood samples were first washed twice with 0.9% NaCl by centrifugation at 3000 rpm for 10 min. Protein was extracted afterward by adding lysis buffer (100 µL DTT 1M, 100 µL Phenylmethylsulfonyl flouride (PMSF) 0.1M, 1 complete tablet, 10 ml T-PER) and centrifuged at 13500 rpm at 4ºC for 15 min, supernatant were recuperated.

In regards to the tissue samples, all of them were weighed and frozen at -80ºC until use. Samples were thawed and 200 µg of each tissue were homogenized in 1 ml of lysis buffer (100 µL DTT 1M, 100 µL Phenylmethylsulfonyl flouride (PMSF) 0.1M, 1 complete tablet, 10 ml T-PER). Tissues homogenized were centrifuged at 13500 rpm at 4ºC for 15 min and supernatants were recuperated.

Protein samples were separated in four aliquots to prevent frizzing and thawing. Before each assay, total protein concentration was determined from each aliquot, spectrophotometrically at 595 nm using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) (38).

Antioxidant enzyme activity

Antioxidant enzyme activity (AEA) was analyzed spectrophotometrically (Thermo Scientific™ GENESYS 10S UV-Vis; Madison, WI USA) in erythrocytes or tissue protein samples as described elsewhere (39). Briefly, SOD activity was determined through the xantine/xantine oxidase system, based on protocols by Paoletti *et al* (40). The superoxide anion formed through this system reacts with the nitro blue tetrazolium (NBT) and generates a formazan salt that was measured spectrophotometrically at 560 nm every 30 seconds for 5 min. One unit of enzymatic activity in this assay is considered as the amount of SOD needed to inhibit 50 % of the superoxide reaction with NBT. CAT activity was quantified using the protocol established by Abei (41), which evaluates the H_2O_2 decrease at 240 nm every 15 sec for 3 min. One unit of catalase activity (UCAT) was considered as the amount of enzyme necessary to catalyze 1μmol of H_2O_2 per minute. GPx activity was analyzed at 340 nm through a protocol described by Ahmad *et al* (42). One unit of GPx activity indicates how much enzyme is required to neutralize H_2O_2 using NADPH (43).

Protein oxidative damage

Carbonyls were determined in order to assess protein oxidative damage by using the DNPH alkaline method (44) and adjusting the optimal volumes for its use in 96 wells plates. Twenty μL of DNPH (10 mM in 0.5 M H_3PO_4) were added to 20 μL of sample protein. Samples were incubated for 10 min in the dark with constant agitation. Afterwards, 10 μL of NaOH (6M)

were added and incubated in the dark for 10 min at room at temperature. Absorbance was determined at 450 nm against a blank where the protein solution was substituted by an equal volume of buffer solution. Carbonyl content was calculated as (Abs_{450}/E)/total protein content of sample. Where E = extinction factor 46.1.

Statistics

A t-student with equal variances was performed to determine differences in antioxidant activity after arousal from torpor, for each tissue. With exception of CAT and GPX activity form lung; and SOD activity from WAT, which didn't have a normal distribution, in which case a U Mann-Whitney test was performed. To determine in which tissue did each enzymatic antioxidant had the greatest activity, a 2-way ANOVA with Tukey pos-hoc was performed. To determine if antioxidant activity was related to the prevention of protein oxidation we performed a one-tail spearman correlation test, between all antioxidant enzymes and carbonyl content during torpor and after arousing.

Results

The results obtained for the antioxidants enzyme activity (SOD, CAT, and GPx) during torpor and arousal, along with protein damage were determined independently in different tissues:

a) Blood

We found a significant increase in antioxidant activity of 2-fold in SOD and CAT, while GPx activity significantly decreased 2-fold after arousal. Carbonyl content showed the same behavior as GPx and significantly decreased 1.3-fold after arousal (Table 1). Furthermore, our results showed a significant positive relationship between SOD and CAT activity during torpor

(r^2 = 0.93, P = 0.0074; Fig 1A); and a positive correlation between carbonyl content during torpor and GPx activity at arousal (r^2 = 0.81, P = 0.0366; Fig. 2A). However, an interesting tendency was found: the more carbonyls found in torpor, the less SOD activity was determined during arousal (r^2 = 0.73, P = 0.0649).

Table 1. Change in antioxidant activity and carbonyl content in bats in torpor and bats at arousal.

	SOD (U/mg)	CAT (U/mg)	GPx (U/mg)	Carbonyls (U/mg)
Blood				
Torpor	1612 ± 346.2	54.88 ± 13.63	4.32 ± 1.11	3.33 ± 0.52
Arousal	3388 ± 894.2	113 ± 19.51	2.00 ± 0.56	2.60 ± 0.11
P	0.0032*	0.0006*	0.0031*	0.0332*
Brain				
Torpor	2069 ± 450.4	59.49 ±6.71	1.76 ± 1.15	0.59 ± 0.09
Arousal	1449 ± 88.86	33.53 ± 11.01	0.46 ±0.26	0.72 ± 0.06
P	0.0165*	0.0020*	0.0396*	0.0932
Lung				
Torpor	359.2 ± 59.12	140.8 ± 16.13	2.56 ± 0.47	1.88 ± 0.61
Arousal	259.4 ± 36.02	103.9 ± 13.75	1.90 ± 0.26	1.93 ± 0.31
P	0.0122*	0.0079*	0.0079*	0.8764
Heart				
Torpor	502.9 ± 58.26	92.09 ± 21.10	2.48 ± 0.33	0.50 ± 0.07
Arousal	571.6 ± 31.86	55.29 ± 4.53	1.76 ± 0.20	0.58 ± 0.06
P	0.0494*	0.0051*	0.0031*	0.058
Liver				
Torpor	576.6 ± 50.87	359.9 ± 47.87	3.95 ± 0.17	0.32 ± 0.19
Arousal	578.1 ± 51.52	302.9 ± 60.86	4.01 ± 0.39	0.31 ± 0.26
P	0.9638	0.1386	0.8306	0.9356
WAT				
Torpor	521.2 ± 228.9	95.4 ± 40.46	0.17 ± 0.54	0.99 ± 0.45
Arousal	414.1 ± 22.32	125.3 ± 26.35	0.46 ± 0.16	1.075 ± 0.24
P	0.5317	0.2037	0.2856	0.7325

Note. Differences between Torpor group and Arousal group are given by the P values; * means significant differences P < 0.05.

Figure 1.- Linear regressions between antioxidant activity and carbonyls content during Torpor.

Comparing variables in torpor (x-axis) against torpor (y-axis) in different Tissues. A) SOD vs CAT in blood; B) SOD vs GPx in brain; C) SOD vs GPx in heart; D) GPx vs CAT in the liver; E) SOD vs CAT in WAT; and F) GPx vs Carbonyls content in WAT. Only significant regressions were plotted ($P < 0.05$)

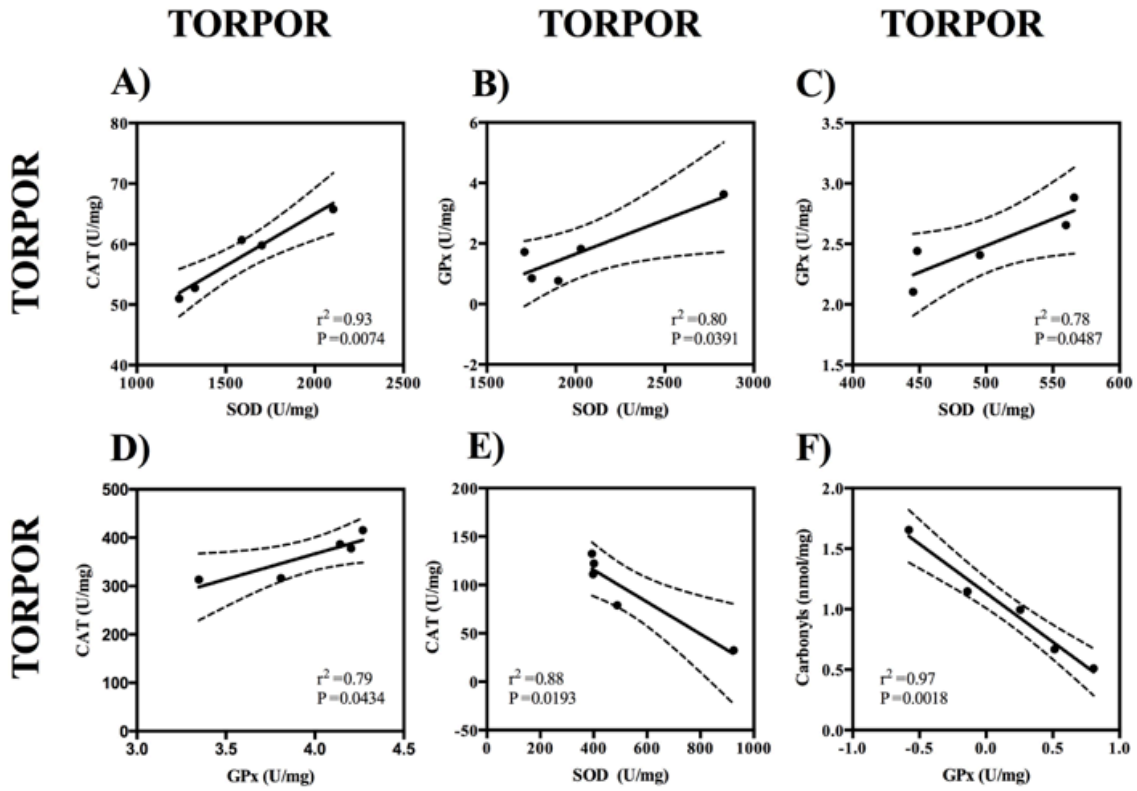
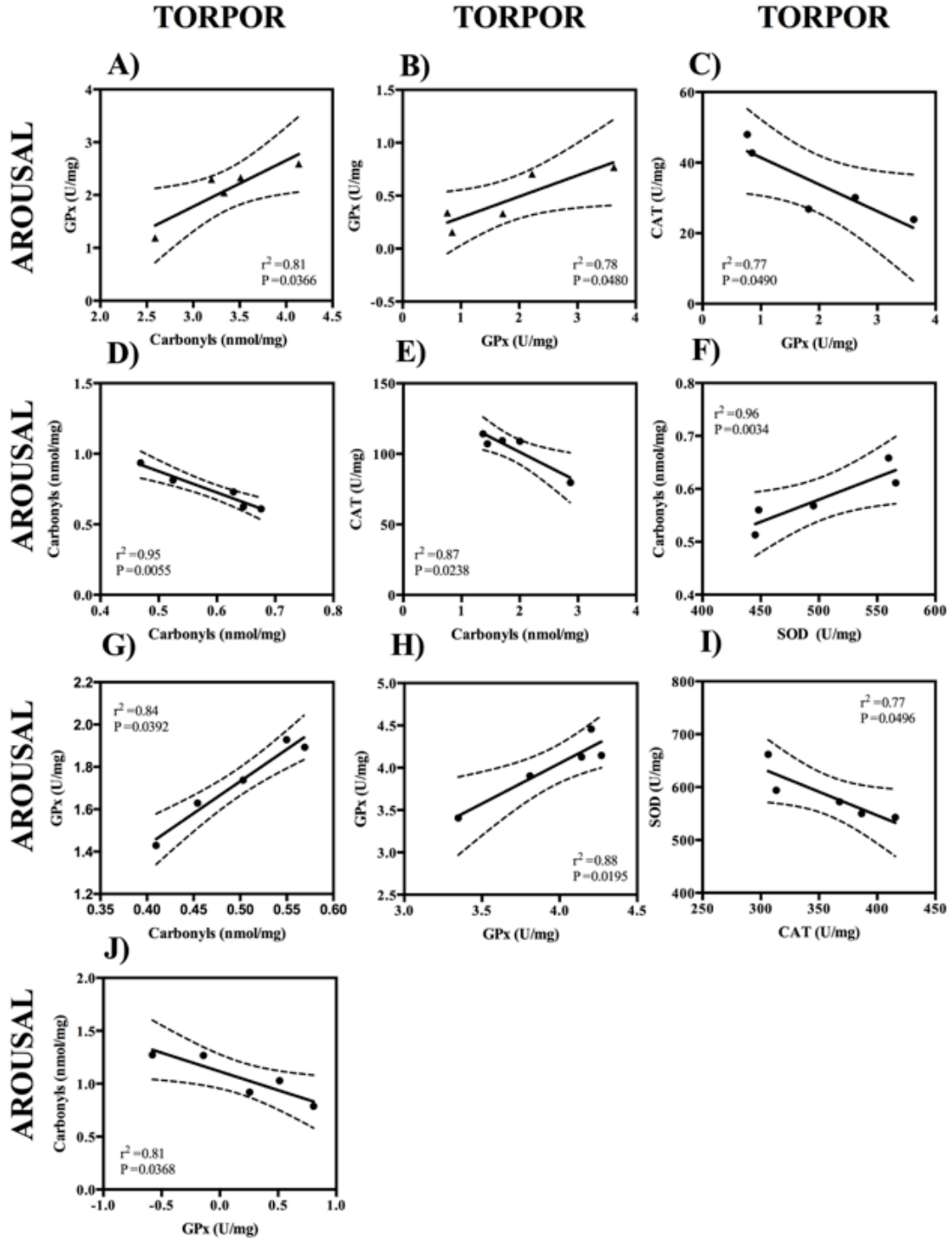


Figure 2.- Linear regressions between antioxidant activity and carbonyls content during Torpor and Arousal.

Comparing variables in torpor state (x-axis) against aroused state (y-axis) in different Tissues. A) Carbonyls content vs GPx in blood; B) GPx vs GPx in brain; C) GPx vs CAT in brain; D) Carbonyls content vs Carbonyls conntent in the brain; E) Carbonyls content vs CAT in the lung; F) SOD vs Carbonyls content in the heart; G) Carbonyls content vs GPx in the heart; H) GPx vs GPx in the liver; I) CAT vs SOD in the liver and J) GPx vs Carbonyls content in the WAT. Only significant regressions were plotted ($P < 0.05$)



b) Brain

All antioxidant enzymes significantly decreased their activity after arousal (SOD by 1.4 fold, CAT by 1.8 fold, and GPx by 3.8 fold), with no change in carbonyls content (Table 1). Also, results showed that SOD activity was positively related to GPx activity during torpor (r^2 = 0.80, P = 0.0391; Fig. 1B). Moreover, GPx activity during torpor was positively correlated to GPx aroused activity (r^2 =0.78, P = 0.0480 Fig. 2B) and negatively correlated to CAT aroused activity (r^2 = 0.77, P = 0.0490; Fig. 2C). Although, we found no direct relationship between protein damage and antioxidant activity, oxidative stress is being prevented, since the more carbonyls there were during torpor, the less there were after arousing (r^2 = 0.95, P = 0.0055; Fig. 2D), in agreement with the finding of no change in protein damage after arousing from torpor.

c) Lungs

Similarly to the brain, all antioxidant enzymes significantly decreased their activity in the lungs after arousal by 1.3 fold in SOD, CAT and GPx, with no change in carbonyls content (Table 1). However, a negative relationship was found between carbonyls content during torpor and CAT activity during arousal (r^2 = 0.86, P = 0.0238; Fig. 2E).

d) Heart

In this tissue, only SOD activity significantly increased 1-fold after arousal, while CAT and GPx activity decreased significantly 1.7-fold and 1.4-fold respectively. No significant change in carbonyls content was observed, but there was a tendency for it to increase (Table 1).

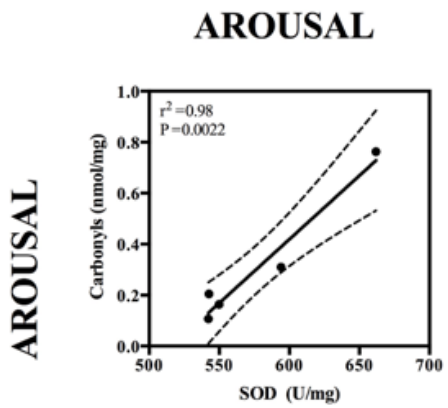
There was a positive relationship between SOD and GPx activities during torpor (r^2 =0.78, P = 0.0487; Fig. 1C). While a positive relationship between SOD activity at torpor (r^2 =0.96, P = 0.0034; Fig. 2F) and carbonyls at arousal was found; however, this was compensated by the

positive relationship between carbonyls at torpor and GPx activity at arousal (r^2 =0.84, P = 0.0392; Fig. 2G).

e) Liver

There were no changes in antioxidant activity or carbonyls oxidation between torpor and arousal (Table 1). However, GPx activity was positively correlated to CAT during torpor (r^2 =0.79, P = 0.0434; Fig. 1D) and to GPx activity at arousal (r^2 = 0.88, P = 0.0195; Fig. 2H). A negative relation between CAT activity at torpor and SOD activity at arousal (r^2 = 0.77, P = 0.0496; Fig. 2I); and a positive relation between SOD activity at arousal and carbonyls content at arousal (r^2 = 0.98, P = 0.0022; Fig. 3) was found.

Figure 3.- Linear regressions between antioxidant activity and carbonyls content during Arousal. Comparing variables in aroused state (x-axis) against aroused state (y-axis). SOD vs carbonyls content in the liver. Only significant regressions were plotted (P < 0.05).



f) WAT

Similarly to liver, there were no changes in antioxidant activity or carbonyls oxidation between torpor and arousal (Table 1) in WAT. Nevertheless, SOD activity was negatively correlated to CAT during torpor (r^2 = 0.88, P = 0.0193), and GPx activity during torpor had a

negative relation with the carbonyl content at arousal (r^2 = 0.97, P = 0.0018; Fig. 1F), and with carbonyls content during torpor (r^2 = 0.81, P = 0.0368; Fig. 2J).

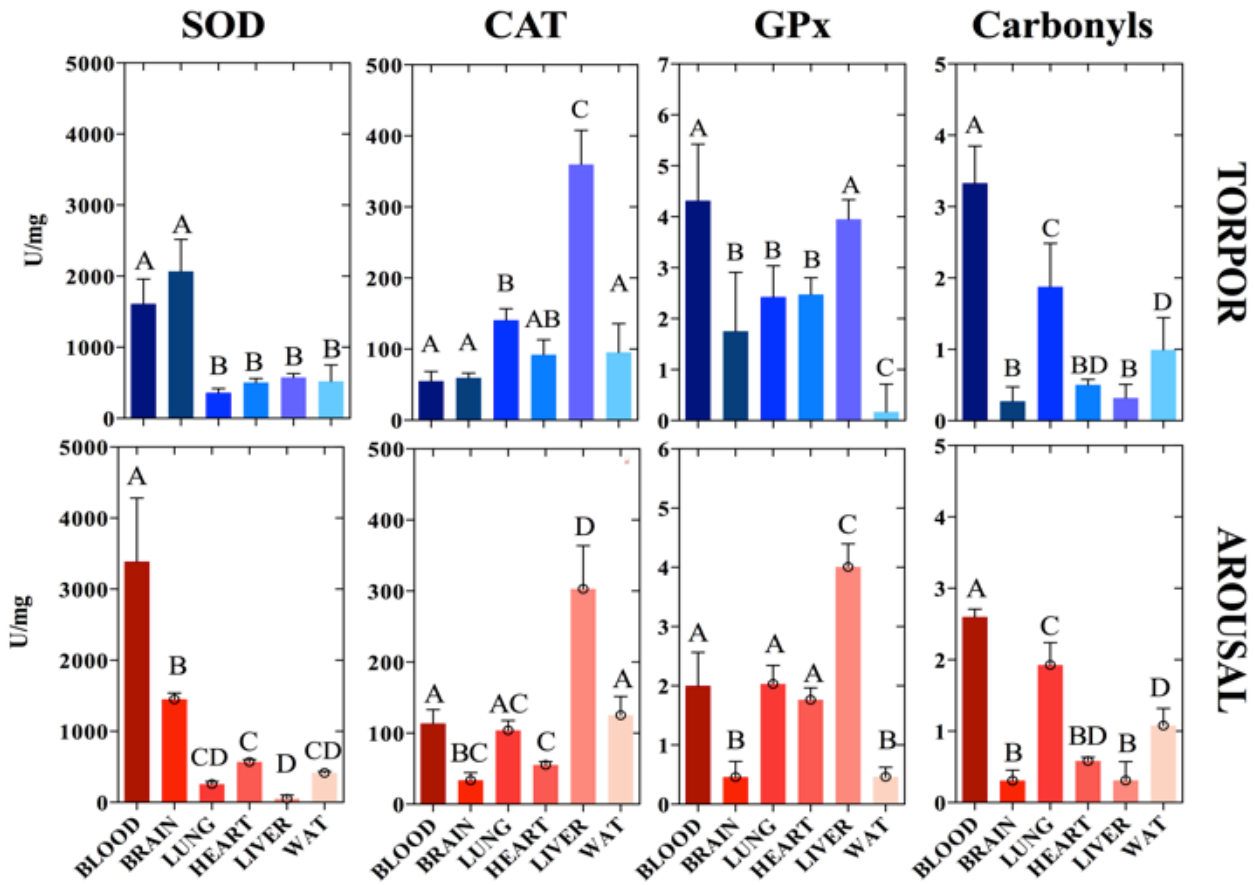
Comparison between organs

When comparing the enzymatic activity among organs, we found that during torpor SOD had the highest activity ($F_{4,48}$ = 85.57, P <0.0001), in the blood and brain. CAT activity ($F_{5,48}$ = 128, P > 0.0001) was predominant in the liver, followed by the lung to a lesser extent, being highest than blood, brain and WAT. While GPx activity ($F_{5,48}$ = 50.77, P < 0.0001) was highest in blood compared to the brain, lung, WAT and heart; liver had the next highest GPx activity when compared to the brain, WAT, lung and heart.

In regards to carbonyl content ($F_{5,48}$ = 115.1, P < 0.0001) during torpor, the highest prevalence was found in blood, followed by the lung when compared to the brain, heart, liver and WAT; and finally followed by WAT when compared to the brain and liver (Fig 4). When arousing, SOD activity remained the highest in blood, brain and heart, respectively ($F_{4,48}$ = 85.57, P < 0.0001). CAT activity remained as the principal antioxidant enzyme ($F_{5,48}$ =128, P > 0.0001) in the liver, but its activity dropped in the lung. Similarly, GPX activity continued high in liver ($F_{5,48}$ = 50.77, P < 0.0001) but dropped significantly in brain compared to the rest of the organs. Carbonyls content ($F_{5,48}$ = 115.1, P < 0.0001) persisted high in the blood, the lung and WAT respectively (Fig 4).

Figure 4.- Antioxidant enzymatic activity and carbonyls content in different tissues during the determined metabolic states.

Comparisons of antioxidant activity and carbonyls content between the different tissues were tested with an ANOVA and Tukey post-hoc. Different literals represent significant differences ($P < 0.05$) between tissues per variable.



Discussion

Hibernation is a physiological adaptation characterized by prolonged torpor bouts, which involves profound decreases in metabolism, heart rate, respiration, and body temperature. Mammalian hibernators undergo repeated cycles of torpor and arousal (i.e. hypoxia-reoxigenation), and several biochemical and physiological parameters are reversed to normal euthermic levels without obvious damages when they return to the aroused state, hence exhibiting high resistance to oxidative stress (3). In many animals, cellular oxidative stress

resistance is associated with enhanced expression of intracellular antioxidant enzymes (16). Here we proved the hypothesis that hibernator bats would be resistant to oxidative stress by up-regulating antioxidant enzymes. We tested a wide range of tissues (blood, brain, lung, heart, liver and WAT) in order to further understand the mechanisms undergoing in stress resistant animals.

We found that antioxidant activity decreased in most organs after arousal; CAT in brain, lung and heart; SOD in brain and lung; and GPx in virtually all organs. Even though, CAT and SOD activity actually increased after arousal in blood, no evidence of oxidative damage was found in the sampled organs. Moreover, we even found a decrease in protein damage in blood after arousal.

This diminished antioxidant activity after arousal could be indicating that the oxidative burst is happening at the early arousal phase, before body temperature reaches euthermic ranges. This is principally supported by the global rise of antioxidants activities in blood after arousal, which suggests that extracellular antioxidants are circulating as a faster defense mechanism while intracellular transcriptional regulation resumes. Also, the positive correlation found in blood, between carbonyls at torpor and GPx activity at arousal, and the negative correlation tendency between carbonyls at torpor and SOD activity at arousal; could suggest that SOD is actively scavenging super oxide radical to H_2O_2 at early arousal before our time sample, at which point GPx activity increased to scavenge H_2O_2. Which is congruent with the high activity of SOD and GPx in blood.

Furthermore, organs seem to relay on different antioxidants for protection, as suggested in our findings. SOD, which catalyzes the super oxide ($O_2^{\bullet-}$) radical into H_2O_2, seems to be the primary antioxidant in the brain and blood, while detoxification of H_2O_2 is supported directly by CAT, which converts two H_2O_2 molecules into H_2O, was found to have the highest activity

in the liver followed by the lungs; and by the catalyzes of GPx of one H_2O_2 molecule into H_2O, found to have the greatest activity in blood and liver. Likewise, organs presented different susceptibility to oxidative stress, the organs that had the highest carbonyls content were blood, lung and WAT; while liver, brain and heart were more resistant and presented lower protein damage.

There are reports that ischemia-reperfusion injury (tissue damage caused when blood supply returns to tissue [reperfusion] after a period of ischemia or lack of oxygen [anoxia or hypoxia]), is similar to the physiological changes that occur during torpor-arousal cycles of hibernation (3). And there are reports that in the brain, after ischemia-reperfusion injury, SOD and CAT activity are up-regulated (45), and deficiency in CuZn–Superoxide dismutase or mitochondrial Mn–Superoxide dismutase exacerbates ischemic brain damage (46). Similarly, our results revealed a high SOD activity in brain, which was positively correlated to GPx activity. However we found no direct relationship between the antioxidant activity and the prevention of protein damage. Again, a possible explanation for this could be due to the effect of our time sampling, as there is evidence that hibernators have regulatory and protective processes against oxidative damage linked to antioxidants transcriptional factors.

AKT (Protein kinase B) activation, render cells more susceptible to ROS-mediated premature senescence and cell death by increasing oxygen consumption and suppressing FOXO activity (which stimulate the transcription of genes coding for antioxidant proteins located in different subcellular compartments (47)), acting as a pro-apoptotic factor under ROS stress (48,49). During hibernation, AKT is repressed in the brain (21), suggesting that during hibernation, FOXO would actively stimulate transcription of its target genes, which include those encoding antioxidant enzymes. (16). Furthermore, levels of active phosphorylated AKT, were found to be strongly reduced in bats´ brain, kidney, liver, and WAT, during torpor as compared with

aroused animals (20). Suggesting that there is an active antioxidant transcription during torpor in thus organs, which in our study (brain and liver) exhibited great resistance to oxidative damage. Also supporting the fact that we found more antioxidant activity during torpor than in arousal. A recent work in two species of hibernating bats, measured lipid peroxidation and antioxidants protein expression on brain, found that SOD protein expression increased after 24 h of arousal, while CAT and GPx protein expression increased after 2h of arousal (much latter than our sample time point); also there was no change in MDA after arousal in one of the species and in the other there was a decrease in MDA after arousal (50). Which indicates that transcription of antioxidants is not immediate and may resume while euthermic hours after arousal.

Additionally, it has been reported that during entry into torpor compared to active animals, SOD protein levels are high in parallel with elevated Nrf2 (a transcript factor involved in the cellular defense against oxidative stress, which controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants (51), and that they decrease gradually as animals fully arouse in heart, BAT and liver of 13-lined ground squirrels; suggesting that induction of synthesis of these antioxidant proteins is triggered as one of the first events when animals begin to suppress metabolic rate (15). This highlights the possibility that hibernators increase antioxidant defenses as an anticipatory or preparatory response in order to deal with the oxidative stress that occurs during prolonged torpor and arousal.

However, the lack of evident oxidative damage in organs after arousing from torpor, seem not to always be due to the action of antioxidants. In the lungs, there was a positive relationship between GPx activity during torpor and carbonyls during arousal; and a negative correlation between carbonyls at torpor, and CAT activity at arousal, implying that antioxidants were not

successfully protecting against oxidative damage. But since there were no significant changes in the amount of carbonyls between torpor and arousal, there is still a possibility that the effect was given due to repair mechanism rather than antioxidant mechanisms. Similarly, in liver, the increased SOD activity along with the maintenance in carbonyl content after arousing from torpor, suggests an enhancement in antioxidant activity to cope with the oxidative stress, but it is highly probably that other mechanisms are also participating. Similarly, in ground squirrels, it has been reported no change in MDA (as measure of lipid peroxidation) in liver and lung after arousal from torpor (27). Nor in carbonyls and TBARS (as measure of lipid peroxidation) during torpor, arousal or active ground squirrels (7). Which reinforces the hypothesis that hibernators have high resistance to oxidative stress.

Contrary to liver and lungs heart and WAT, exhibited a high correlation, which linked GPx activity (which also was the predominantly antioxidant activity in thus tissues) with less carbonyls content.

White adipose tissue has three main functions: heat insulation, mechanical cushion, and most importantly, a source of energy. Furthermore, it has also been recognized as a critical endocrine organ, which secret a number of important proteins, i.e. leptin which plays a pivotal role in the regulation of energy balance (52). Making WAT an important ROS target for lipids and proteins oxidation. There is not much information available regarding antioxidant protection during hibernation for WAT. In ground squirrel there is a report of an increase in CAT protein expression in torpid animals compared to active (at room temperature) animals (17). In our study antioxidant activity (principally CAT and GPx) did not differ between torpid or aroused animals in WAT, but according to such report, it appears they have more antioxidants than active summer animals, and in our study there is a clear relationship between antioxidant activity preventing protein damage.

Likewise, there is not much information available in antioxidant defense for heart, but interestingly, there is a report of an increase in protein expression of all three peroxiredoxin enzymes (Prdx1, Prdx2, Prdx3), which reduce and detoxify a wide range of hydroperoxides, in torpid ground squirrels, compared to active animals. Highlighting the importance of peroxide scavengers in this tissue, as GPx and CAT were the primary antioxidants activated in our study. Besides, GPx was directly related with protein damage protection. Furthermore, there has also been suggested that antioxidant protection is reduced the longest the animal stays in a torpid state. Thus, animals arousing from a full season of hibernation, during which I-R stress would be expected to be high, would not have enhanced antioxidant capacities in the major oxidative tissues (16). However, further research in this topic should be prioritized, as multiple interruptions on animals' hibernation could severely deplete antioxidant defenses before the hibernation season ends.

Our study suggests that hibernating bats are being thoroughly protected against oxidative damage treat at the moment of arousal, and that the antioxidant enzymes are related to such protection at least in some tissues. Nevertheless, further research at different time points, as well as repair mechanism are needed (53).

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CONCLUSIONES GENERALES

El objetivó general de este trabajo fue determinar si los parámetros fisiológicos evaluados para determinar el estrés (GCs, estado redox, e inmunocompetencia), se relacionaban entre sí y permitían proporcionar un panorama más completo de la respuesta de los animales silvestres frente a cambios drásticos en el medio ambiente.

Lo anterior para estudiar la forma en que los animales, que están evolutivamente adaptados a condiciones ambientales adversas, responden ante estímulos de estrés.

Glucocorticoides e inmunocompetencia

Se encontró que en condiciones basales, los murciélagos con mayores niveles de metabolitos fecales de cortisol (FCM) presentaban menor respuesta inflamatoria, lo cual concuerda con el rol antiinflamatorio de los GCs reportado en literatura (68). Sin embargo, al aplicarles un estimulo de estrés agudo, aumentó la respuesta inmune celular. Lo cual sugiere que el organismo se protege en caso de sufrir alguna herida, como podría ser, por ejemplo, al huir de un depredador. No obstante, cuando el estimulo se prolonga por más de 24 h o la cantidad de GCs liberada es muy elevada, entonces la respuesta inflamatoria vuelve a disminuir. Mientras que la respuesta humoral estuvo siempre relacionada positivamente con los niveles de GCs, tanto en condiciones basales como de estrés agudo. Por lo que nuestros resultados sugieren que la forma en que se ha conceptualizado la relación glucocorticoides-inmunocompetencia, debería ser re-evaluada. El estrés no necesariamente reduce la inmunocompetencia, ya que en algunos casos, promueve algunos componentes de la respuesta inmune. Esto requiere que los investigadores sean muy específicos al analizar la forma, intensidad y duración de el estrés estudiado, y el componente del sistema inmune que está siendo evaluado. Además, los resultados obtenidos en las poblaciones naturales deben ser

interpretados con cuidado, puesto que muchas veces se desconoce a que otros estímulos han sido expuestos los organismos previo a su captura. Nuestros resultados a este respecto sugirieron que cuando un organismo se confronta con un reto inmunológico al mismo tiempo que con un estímulo de estrés fuerte, disminuyeron los niveles de GCs, bajando su reactividad al estrés. Por lo que se debería priorizar más estudios al respecto.

Glucocorticoides y estrés oxidativo

Es importante resaltar que algunos de los estudios que han reportado que los glucocorticoides provocan estrés oxidativo, no realizaron un revisión a mayor profundidad. En esos estudios solo se presentaron resultados de antioxidantes no enzimáticos, como los carotenoides (69), asociados a un aumento en las ERO al administrar GCs exógenos (70). Sin embargo, el aumento en las ERO, no necesariamente significan que exista un estado de estrés oxidativo, ya que solo medir antioxidantes secundarios únicamente proporciona un panorama muy reducido sobre el estatus antioxidante del organismo.

En esté estudio se encontró una relación positiva entre los GCs y la respuesta antioxidante, tanto en condiciones basales como en los eventos de estrés corto. No obstante, no se encontró ninguna relación con el daño a las proteínas, lo que podría sugerir que los GCs promueven la actividad enzimática antioxidante, al menos a corto plazo.

Así mismo, es relevante mencionar que la respuesta antioxidante ante un potente estimulo de estrés (estimulación con ACTH exógeno) y/o la estimulación con un reto inmunológico, no es la misma para todas las enzimas. La actividad de la CAT aumentó significativamente cuando el organismo fue estimulado con ACTH o con el reto inmunológico. Por su parte, la actividad de la GPx solo aumentó cuando fue desafiada con el reto inmunológico. De manera similar a lo encontrado para la CAT, la actividad de la SOD aumentó con el reto inmunológico, sin embargo cuando era presentado con ambos estímulos al mismo tiempo, su actividad bajó

significativamente. Por lo tanto, la enzima SOD, siendo la primera barrera contra los radicales libres, tiene una función antioxidante importante. Aún así, la CAT mostró tener un papel más evidente en prevenir el daño oxidante durante el estrés mediante la eliminación de peróxido de hidrogeno. Mientras que la enzima GPx aunque tuvo un rol importante para remover H_2O_2, su actividad estuvo mayormente relacionada con la respuesta inmune que con la respuesta al estrés.

Estrés oxidativo y torpor

La respuesta antioxidante evaluada en *M. velifer* estuvo relacionada directamente con la prevención del daño oxidante en algunos órganos como el corazón, el hígado y el TAB. Sin embargo, su relación no fue tan evidente en otros órganos como el cerebro y los pulmones. A pesar de esto, se encontró evidencia que la sangre se encargó de aumentar la circulación de los antioxidantes después de despertar del torpor. Puesto que ningún órgano presentó daño oxidativo en proteínas, lo que sugiere que existan otros mecanismos de protección en juego, además de la respuesta antioxidante. Nuestros resultados también sugieren que la explosión respiratoria que ocurre al despertar del torpor sucede mucho antes que la temperatura corporal llegue a rangos eutérmicos. Por lo que cabe la posibilidad de que se esté generando daño por estrés oxidativo a inicios del despertar, y que probablemente haya mecanismos de reparación de daño en juego.

Se necesitan más estudios detallados sobre la taza de aumento del consumo de oxigeno y producción de las ERO para obtener un mejor perfil de los cambios que ocurren en el estado redox a lo largo del proceso de pasar de estado hipotérmico/hipoxico al estado de eutermia. Aún así, se demostró que la respuesta antioxidante juga un papel importante en la protección contra los eventos de isquemia-reperfusión que ocurren durante ciclo de torpor.

En conclusión, los organismos adaptados a ambientes adversos fueron altamente resistentes al estrés. Los GCs y la actividad enzimática antioxidante aumentaron durante los eventos de estrés agudo sin presentar daño celular; sin embargo, cuando el estrés se prolongó por un periodo más largo de tiempo, o coincidió con otros eventos energéticamente demandantes (como la lactancia, o algún reto inmunológico), la eficiencia en la respuesta al estrés se vio comprometida.

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