Universidad Autónoma de Querétaro
Facultad de Ciencias Naturales
Doctorado en Ciencias Biológicas

Hematología clínica como indicador de salud y cambios ontogénicos en el lobo marino de California (*Zalophus californianus*): Un enfoque temporal y espacial

TESIS INDIVIDUAL

Que como parte de los requisitos para obtener el Grado de
Doctorado en Ciencias Biológicas

Presenta:
Adriana Estefanía Flores Morán

Dirigido por:
Karina Alethya Acevedo Whitehouse

SINODALES

Dra. Karina Alethya Acevedo Whitehouse Presidente	Firma
Dr. Fausto Arellano Carbajal Secretario	Firma
Dr. Rolando Tenoch Bárcenas Luna Vocal	Firma
Dra. Milagros González Hernández Suplente	Firma
Dra. Laila Pamela Partida Martínez Suplente	Firma

C.S. Juana Elizabeth Elton Puente
Directora de la Facultad de Ciencias Naturales

Dra. Ma. Guadalupe Flavia Loarca Piña
Directora de Investigación y Posgrado

Centro Universitario
Querétaro, Qro.
Enero 2019
México

DECLARATORIA DE AUTORÍA

Declaro que el trabajo que se presenta en esta tesis fue realizado por mí, a excepción de aquellas secciones que formaron parte de colaboraciones, mismas que se indican explícitamente en el texto.

Confirmo que las ideas originales aquí presentadas son de mi autoría y que a lo largo de la tesis le he dado crédito al trabajo de otros mediante el uso adecuado de las referencias.

Esta copia se ha sometido a la Universidad Autónoma de Querétaro bajo el entendido de que constituye material con derechos de autor y que no puede citarse ningún pasaje de esta tesis sin darle el crédito adecuado.

RESUMEN

El descubrimiento de Leeuwenhoek fue un parteaguas para la investigación de elementos que no podemos observar a simple vista, pero que son esenciales para la biología. Unos de estos elementos microscópicos son las células de la sangre, las cuales hoy en día se sabe que participan en funciones vitales para el organismo. Además, el análisis de estos elementos figurados de la sangre es indispensables para evaluar el estado de salud de los individuos. En la presente tesis se realizó un análisis de las células sanguíneas de crías de lobo marino de California (*Zalophus californianus*) desde una perspectiva ontogenética y ecológica, que sirviera para generar información de base para especies de vida libre. También se exploró si eventos anómalos climatológicos, que resultan en restricciones alimenticias de las madres, impactan procesos fisiológicos como la síntesis y maduración de las células sanguíneas, así como la abundancia de diferentes poblaciones leucocitarias en las crías de lobo marino de California. Los resultados de esta tesis son esenciales para entender el impacto y las posibles consecuencias de eventos de cambio climático en especies de vida libre. Se presenta la tesis en el formato de los artículos publicados, que derivaron de este estudio, conforme a los lineamientos del programa del Doctorado en Ciencias Biológicas de la Universidad Autónoma de Querétaro.

Palabras clave: hematología, lobo marino de California, desarrollo

ABSTRACT

Leeuwenhoek’s invention set the ground for the beginning of the exploration of elements that we cannot see in plain sight but that are essential to biology. Some of these microscopic elements are blood cells, which are known to be involved in essential body functions, homeostasis and health. Interestingly, there is limited information about changes in blood cell morphology for many free living species. This thesis examined changes in red and white blood cells of California sea lions (*Zalophus californianus*) from an ontogenetic and ecological perspective. I examined variations in blood cell morphology along pup development, and across their distribution in the Gulf of California. Another one of my goals was to understand how anomalous climatic events that impact feeding patterns in adult sea lion females can affect physiological process in pups, such as the synthesis and development of red blood cells, and also how it can influence white blood cell counts of these animals. The results of this thesis are important to understand the effects and possible consequences of climate change for wildlife from a physiological perspective. The thesis is presented in the format of the scientific manuscripts that derived from this study according to the Doctoral program of the Universidad Autónoma de Querétaro.

Key words: hematology, California sea lion, development

DEDICATORIAS

Para Alí, Nabil y José Luis

AGRADECIMIENTOS

Esta tesis fue financiada con el Proyecto 446 de Fronteras de la Ciencia-CONACYT.

A CONACYT por la beca otorgada del 2014 al 2018 para la realización del doctorado con número de becario 287579

A Alí y a Nabil, porque darme cuenta de lo que realmente es importante en la vida me ha dado la seguridad y la fortaleza de abrirme paso en la vida y e ir detrás de mis sueños. Los amo.

A Karina, siempre te agradeceré el haberme dado un lugar en tu equipo de trabajo y por tenerme tanta paciencia y aunque a veces el panorama fuera muy sombrío siempre creíste en mí.

A mis sinodales Fausto, Rolando, Mila y Laila, por semestre con semestre escucharme y darme buenos consejos.

A mi mamá, gracias porque se que a pesar de todo lo que tenias en contra siempre trataste de darme lo mejor de ti. Y muchísimas gracias por cuidar siempre de mi chamis y de toda la pandilla.

A la señora Bertha y a Don Raúl por apoyarme tanto como a una hija, muchas gracias.

A mis hermanos por apoyarme con cuanto han podido, sobre todo con risas. Gracias por ayudar a cuidar a la chamis y el resto de la pandilla.

A los equipos de polibikes (Arge, Charlie, Kika, Mau) y de lima lama (Profe Carlos), que de verdad no saben como estar con ustedes me ayudo a sobrellevar todo el estrés del día a día.

A Chava Padilla por haberme recibido en tu laboratorio y contagiado de tu pasión por la hematología clínica y a Luis Enrique porque tu sencillez y agradecimiento con la vida y con las personas que te rodean me enseño mucho y por siempre recibirme y ayudarme con mi trabajo.

Al laboratorio de Plasticidad Inmune y Ecoepidemiología Molecular, gracias por esos momentos tan divertidos y por aceptarme con todo y mi pasado pandillero jajajaja.

A Ale Herbert, gracias por las horas y horas de platicas y risas, tu mas que nadie me ha enseñado como ser un buen científico.

Al Dr. Mauricio Díaz, Dra. Olivia Vázquez y Dra. Cris Medina por el curso de Creadores de la Ciencia Moderna que en mi opinión debería ser materia obligatoria en todos los programas de posgrado, el cual me recordó el porque estoy en el ambiente científico y a disfrutar y amar lo que hago. Muchas gracias por el libro.

A Pili muchas gracias por tu amistad sin duda alguna una de las mas sinceras.

A Rocío y Yara gracias por recorrer conmigo este camino desde los 15 años y por esas tardes de chisme en los tacos ahogados jajaja.

A Damian mi gran amigo de toda la vida,

A Gloria, gracias por siempre tener un espacio en tu día para mi Alí.

A Cele, Brenda, Rebe, Val y por supuesto a Kim, por su gran apoyo con Alí en los momentos mas difíciles y por hacerlo sentir siempre como parte de su familia.

A Concha, Nirvana, Wendy y Tonalli por recordarme lo valiosa de la infancia, de la humildad, el amor y respeto que se necesita para guiar a un niño. Sin duda un aprendizaje que guardo valiosamente en mi corazón.

A José Luis, por dejame ser parte de tu vida, por todo lo que hemos aprendido juntos y por ese enorme y noble corazón que tienes. Gracias por darme siempre ese empujoncito para alcanzar mis metas.

“La principal y más poderosa rémora que detiene a nuestro país en el camino del engrandecimiento es la ignorancia; la falta de ilustración de nuestro pueblo es la que lo convierte en pasivo e inconsciente instrumento de los intransigentes y parlanchines que lo explotan sin cesar, haciéndolo a la vez víctima y verdugo de sí mismo”
— Gabino Barreda

Contenido

INTRODUCCIÓN

La hematología, o estudio de la sangre, es una rama de la biología que es comúnmente utilizada en estudios clínicos de humanos y animales, tanto domésticos como silvestres. Resulta interesante que, en contraste con las investigaciones enfocadas en la salud, se ha dejado a un lado el estudio de la hematología desde los puntos de vista ontogénicos, fisiológicos y ecológicos, particularmente para las poblaciones silvestres. **En esta tesis se investigaron patrones de variación temporal y espacial de diversos parámetros hematológicos en una población silvestre del lobo marino de California, *Zalophus californianus*, en el Golfo de California, y se pusieron a prueba diversas hipótesis sobre el desarrollo temprano de los individuos, diferencias inter-sexo y asociaciones con índices de condición y salud, en un contexto ecológico.**

La sangre es un tejido constituido por elementos celulares, que en conjunto reciben el nombre de ‘elementos figurados de la sangre’, y por una matriz extracelular líquida llamada plasma sanguíneo compuesta por agua, sales minerales y proteínas (Sepulveda, 2014). Las células sanguíneas comienzan a formarse desde las etapas tempranas de la gestación, en sitios activos de la hematopoyesis como el saco vitelino, el hígado y la médula ósea (Proytcheva, 2009).

Las células de la sangre participan en diversas funciones; los **leucocitos** participan en respuestas inmunes, tanto innatas como adquiridas (Tizard, 2009); los **eritrocitos** tienen la capacidad de plegarse para pasar a través de todos los tejidos en donde regulan los niveles de oxígeno y dióxido de carbono, además de participar en la regulación del metabolismo del óxido nítrico, regulación redox, reología de la sangre y viscosidad (ver Kuhn et al., 2017); y las **plaquetas** que en realidad son fragmentos de megacariocitos que contienen proteínas en su superficie que les permiten adherirse al endotelio y entre ellas cuando ocurre una pérdida de la continuidad endotelial, por lo que participan en la hemostasia y coagulación (Holinstat, 2017).

Los efectores sanguíneos son de suma importancia para los organismos, debido a las múltiples y variadas funciones en las que participan y a su estrecho contacto con células y tejidos del organismo, por lo que variaciones en el estado fisiológico pueden ser reflejadas en la sangre. Por esto, en el área clínica, el análisis de la sangre es muy importante para la evaluación de la salud y detección de diversas patologías (Nuñez y Bouda, 2009). Sin embargo, su análisis también puede ayudar a identificar y entender diversos procesos fisiológicos, como la transición entre el ambiente uterino y el extrauterino, la maduración de órganos hematopoyéticos, la anemia fisiológica del recién nacido y deficiencias nutricionales (Hillman et al., 2012; Proytcheva, 2009; Ruef and Linderkamp, 1999; O’Brien 1971).

A diferencia de los estudios hematológicos realizados en mamíferos terrestres de vida libre, son escasos los estudios realizados en mamíferos marinos. Sin embargo, algunos, han reportado que los valores hematológicos de la foca de puerto (*Phoca vitulina*), foca de Groenlandia (*Phoca groenlandica*), foca de casco (*Cystophora cristata*), delfín nariz de botella (*Tursiops truncatus*) y lobo marino de Steller (*Eumetopias jubatus*) varían de acuerdo al sexo, edad, condición corporal y maduración sexual (ver Boily et al., 2006; Fair et al. 2006; Greig 2010; Skinner et al. 2015). Aunque menos frecuentes aún, también se han realizado estudios sobre alteraciones morfológicas de las células sanguíneas de algunos mamíferos marinos (Clark et al., 2002), y se ha comenzado a explorar la fisiología hemática. Por ejemplo, un estudio reciente demostró que es posible detectar en belugas (*Delphinapterus leucas*) de dos meses de edad uno de los procesos fisiológicos que han sido bien descrito en el humano, llamado “anemia fisiológica” (Noren et al., 2018). Sin embargo, la gran cantidad de información relacionada con salud y desarrollo que se puede obtener a partir del tejido sanguíneo aún es escasa para estas especies.

Los mamíferos marinos son considerados como especies centinelas del ambiente ya que pertenecen a niveles tróficos altos, poseen una capa gruesa de grasa, son longevos, y tienen una gran variedad de distribuciones y patrones de movimiento, que reflejan aspectos de productividad del océano, variaciones en la cadena trófica, acumulación de contaminantes y la aparición de enfermedades emergentes (Bossart, 2011; Moore, 2008).

En este mismo sentido, los mamíferos marinos son modelos interesantes para estudiar la ontogenia hemática. Las adaptaciones que presentan estas especies en el tejido sanguíneo les confieren una gran capacidad para el buceo, lo que requiere de un gran aporte de oxígeno, obtenido principalmente de los eritrocitos. Además, la producción de células sanguíneas requiere de elementos indispensables como hierro (Naigamwalla y Webb, 2012), folatos y vitaminas, como la B12 (Koury y Ponka, 2004), hormonas, como la eritropoyetina (EPO; Föller et al., 2011; Lombardero et al., 2011; Kling, 1996), mediadores inmunes (Rodak, 2002) y variaciones en la concentración de oxígeno en los tejidos. Es por esto que alteraciones en estos procesos fisiológicos son reflejados en la sangre.

Resulta interesante el que los efectores de la sangre no siempre responden de la misma forma en los individuos. Por ejemplo, estudios realizados en el lobo marino de California (*Zalophus californianus*) y el lobo marino de Galápagos (*Zalophus wollebaeki*) reportaron que la infiltración de células inflamatorias ante un estímulo dado es dependiente del contexto y de la etapa de desarrollo de las crías (Brock et al., 2013; Vera-Massieu et al., 2015). Por su parte, en el lobo marino de California la respuesta inflamatoria es dependiente de la condición corporal de las crías durante las etapas más tempranas, y en etapas posteriores del desarrollo depende de procesos infecciosos concomitantes (Vera-Massieu et al., 2015).

El lobo marino de California, utilizado como modelo de estudio en esta tesis, se distribuye desde Vancouver, en el Pacífico noreste, hasta el Golfo de California en México, en donde habita en 13 colonias reproductivas. Es una especie filopátrica y poligínica que presenta una temporada reproductiva anual. Los individuos presentan un marcado dimorfismo sexual (Odell, 1975), siendo los machos de mayor peso y tamaño, con una estructura ósea en el cráneo llamada cresta sagital. Tanto hembras como machos poseen una gruesa capa de grasa (King, 1983). La inversión materna es marcada, con un periodo de lactancia que dura al menos seis meses, periodo en el que las crías no reciben ningún otro alimento (King, 1983). En términos ecológicos, este otárido pinnípedo es un depredador tope con hábitos costeros generalistas (Lowry et al., 1990; García-Rodríguez y Aurioles-Gamboa, 2004). Las hembras adultas se alimentan en un radio de 50-100 km de la isla reproductiva que habita (Kuhn y Costa, 2014). La mayor parte de la información obtenida sobre los hábitos alimenticios de la especie se han basado en el análisis de las estructuras óseas auditivas de las peces (otolitos); aunque más recientemente se ha incorporado el análisis de isótopos estables ($\delta^{13}C$, $\delta^{15}N$), lo que ha permitido identificar las zonas de alimentación y el nivel trófico en el que se alimentan estos mamíferos (Elorriaga-Verplancken et al., 2016). En conjunto, estas características hacen del lobo marino de California un modelo ideal para entender cómo los procesos fisiológicos son afectados por el crecimiento, disponibilidad de recursos y estado de salud, en un contexto ecológico.

El estudio de la sangre de los individuos también podría ser relevante para comprender los efectos de los cambios climáticos sobre la fisiología de los individuos. Una de las áreas que ha tenido un mayor interés en los últimos años es el efecto del cambio climático en las especies en términos de abundancia, reproducción (Blaustein et al., 2002; Jenssen, 2006), comportamiento, endogamia, conflictos con asentamientos humanos, etc. (ver Parmesan, 2006). Sin embargo, los efectos del cambio climático sobre la fisiología y la salud de los organismos silvestres durante eventos de cambio climático han sido poco explorados. También en este sentido resulta ideal un modelo de estudio como el que representan los mamíferos marinos, en particular las especies costeras de ámbito hogareño reducido como el lobo marino de California (Bossart, 2011; Moore, 2008).

Uno de los eventos recientes de variación climática tuvo lugar durante el invierno del 2013 en Alaska (Bond et al., 2015), en donde la temperatura superficial del mar tuvo un incremento, evento llamado “La Mancha” (The Blob), que se extendió de manera gradual durante el 2014 y 2015 en el pacífico noroeste (Kintisch, 2015), desde Alaska hasta la Península de Baja California del lado del Pacífico. La anomalía climática empeoró ya que para el 2015 coincidió con otro evento climatológico conocido como El Niño-Oscilación del Sur (ENSO, por sus siglas en inglés), y alcanzó un mayor incremento de temperatura, de hasta 5℃ durante dicho año (National Centers of Environmental Prediction SST Analysis, National Oceanic and Atmospheric Administration, http://polar.ncep.noaa.gov/sst/). El incremento de la temperatura superficial del mar derivado de La Mancha disminuyó la productividad primaria, que impacto en las poblaciones de lobo marino de California, en particular en términos de sus hábitos alimentarios (Elorriaga-Verplancken et al., 2016). En esta tesis se exploraron los efectos de esta anomalía climática sobre la fisiología sanguínea en el lobo marino de

California, y su relación con variables ecológicas, así como su efecto sobre parámetros de salud de los individuos. A partir de estos análisis se generaron dos manuscritos, ya publicados, y un tercer manuscrito, enviado para su consideración, que se presentan en esta tesis.

OBJETIVO GENERAL

Realizar un análisis cuantitativo y cualitativo de diversos efectores sanguíneos de crías de lobo marino de California (*Zalophus californianus*) y establecer valores de referencia para la especie en vida libre, además de integrar el análisis de sangre en un marco ecológico para entender como la morfología y la abundancia de las células de la sangre reflejan condiciones de abundancia de recursos, enfermedades subclínicas, y cambios ambientales.

OBJETIVOS ESPECÍFICOS

1. Realizar un análisis cuantitativo y morfológico de leucocitos, eritrocitos y plaquetas de crías de lobo marino de California en vida libre.
2. Estimar valores de referencia de leucocitos para las crías de lobo marino de California de vida libre.
3. Investigar si las anormalidades en la morfología de los eritrocitos y la abundancia de leucocitos de las crías está determinada por variaciones en las estrategias alimentarias de las madres durante años caracterizados por eventos climáticos anómalos.
4. Determinar cuáles parámetros hematológicos son indicadores ecológicos para el lobo marino de California en vida libre.

ARTICULO I

En esta sección se presenta el primer artículo derivado de la tesis, con título “Atypical Red Blood Cells Are Prevalent in California Sea Lion Pups Born during Anomalous Sea Surface Temperature Events”, que fue publicado en 2017 en Physiological and Biochemical Zoology.

Atypical Red Blood Cells Are Prevalent in California Sea Lion Pups Born during Anomalous Sea Surface Temperature Events

Adriana Flores-Morán[1,*]
Marina Banuet-Martínez[1,*]
Fernando R. Elorriaga-Verplancken[2]
Luis Enrique García-Ortuño[3]
Julieta Sandoval-Sierra[2]
Karina Acevedo-Whitehouse[1,4,†]
[1]Unit for Basic and Applied Microbiology, School of Natural Sciences, Autonomous University of Queretaro, Queretaro 76230, Mexico; [2]Department of Fisheries and Marine Biology, Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional (CICIMAR-IPN), La Paz 23094, Mexico; [3]Laboratory of Clinical Pathology, Department of Pathology, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Coyoacán 04510, Mexico; [4]The Marine Mammal Center, 2000 Bunker Road, Sausalito, California 94965

Accepted 5/15/2017; Electronically Published 7/3/2017

ABSTRACT

To date, there is limited knowledge of the effects that abnormal sea surface temperature (SST) can have on the physiology of neonate pinnipeds. However, maternal nutritional deficiencies driven by alimentary restrictions would expectedly impact pinniped development and fitness, as an adequate supply of nutrients is essential for growth and proper functioning of all body systems, including red blood cell synthesis and clearance. Here, we investigated red blood cell morphology of California sea lion (CSL) pups from the San Benito Archipelago born during the 2014 and 2015 anomalously high SST events recorded in the northeastern Pacific Ocean. We examined whether atypical erythrocyte morphologies were more common in 2015, when the high SST event was more pronounced, and whether the stable isotope signature of pup fur, as an indicator of maternal feeding strategies, accounted for the number of atypical cells. Various atypical erythrocyte morphologies were more prevalent and more abundant than reference values. Evidence of iron deficiency was found in both years, and only pups born in 2014 showed evidence of active erythropoiesis. Microcytes and reticulocytes were more common in pups with higher isotopic $\delta^{13}C$ and lower $\delta^{15}N$ values, suggesting a probable relationship between maternal feeding strategies and the effect of climatic anomalies on red blood cell physiology of their pups. As developing pinnipeds require increased oxygen storage capacity for diving and foraging, the presence of atypical erythrocytes could be relevant to CSL pup fitness if the underlying cause is not reverted. This study is a first step to explore the effects that climatic alterations in the marine environment can have on the blood physiology of developing individuals.

Keywords: anemia, anisocytosis, atypical red blood cells, California sea lion, El Niño, microcytes, reticulocytes, SST, "the Blob."

Introduction

During the boreal winter of 2013, anomalous sea surface temperatures (SST) were recorded in the Gulf of Alaska (Bond et al. 2015). This unprecedented event, termed "the Blob," raised concern as the high SST spread southward, encompassing the entire northeastern Pacific (Kintisch 2015). By summer 2015, the Blob had reached the southernmost tip of the Baja California Peninsula, in Mexico, meeting the El Niño 2015 conditions, which further increased the SST (National Centers for Environmental Prediction SST Analysis, National Oceanic and Atmospheric Administration, http://polar.ncep.noaa.gov/sst/). Consequences of the climatic anomaly for the ocean's primary productivity were quickly evident, with reductions in fish abundance and an increase in stranding events (Chust et al. 2014; McClatchie et al. 2016). However, the effects of these simultaneous anomalous SST on the physiology of marine organisms, particularly long-lived predators, largely remain unexplored.

The California sea lion, *Zalophus californianus* Lesson 1828 (hereafter, CSL), is distributed in the northeastern Pacific along the area affected by the high SST. This otariid pinniped is a generalist feeder with coastal habits (Lowry et al. 1990; García-Rodríguez and Aurioles-Gamboa 2004) whose feeding range tends to be within 50–100 km from its breeding islands (Kuhn and Costa 2014). The species' coastal foraging habits have been inferred through the stable isotope of carbon ($\delta^{13}C$), which tends to reveal relatively high values (Elorriaga-Verplancken et al. 2016*b*) because of phytoplankton blooms and the presence of ^{13}C-enriched macrophytes in that ecosystem baseline (Michener and Schell 1994; France 1995). The stable isotope of nitrogen ($\delta^{15}N$) is also useful to understand the trophic ecology of consumers, due to the fact that its values and individuals' trophic

---

[*] These authors contributed equally to the study.
[†] Corresponding author; e-mail: karina.acevedo.whitehouse@uaq.mx.

Physiological and Biochemical Zoology 90(5):564–574. 2017. 1522-2152/2017/9005-7033$15.00. DOI: 10.1086/692919

position tend to show a positive relationship (Minagawa and Wada 1984).

As they are coastal feeders, diminished prey availability secondary to the high SST greatly affects CSLs, which are forced to either extend their foraging trips to procure prey (Kuhn and Costa 2014; Elorriaga-Verplancken et al. 2016*b*) or consume other prey, often of lower nutritional and energetic value, giving as a result a recent high number of early weaned CSL pups stranded in poor condition in Southern California (McClatchie et al. 2016). According to the life-history trade-off hypothesis (Stearns 1992), the cost of procuring prey under high SST conditions would be highest for pregnant or nursing adult female California sea lions, as they are already experiencing elevated energetic demands. Maternal nutritional deficiencies would expectedly impact on CSL development and fitness, as an adequate supply of nutrients is essential for growth and proper functioning of all body systems (Wu et al. 2016). Evidence of negative effects caused by El Niño events have already been known to hamper the development of sea lion pups, which were reported to grow at a lower rate and have increased mortality (Boness et al. 1991; Ono et al. 1993; Melin et al. 2010).

If CSL pups suckle milk from mothers that were malnourished or energetically impoverished due to the abnormal SST conditions, their development could become impaired. Some indications of this have already been reported. For instance, the mass of CSL pups born in 2014 on the San Benito Archipelago (SBA), off the coast of the Baja California Peninsula, was significantly higher than for those born in 2015 (Elorriaga-Verplancken et al. 2016*b*), when the Blob and El Niño simultaneously caused the most intense SST anomaly in this region. Specifically, on average, the SST anomaly was 1°C higher in 2015 than in 2014 for the central part of Baja California, where the SBA is located, and even exceeded 4°C in some months (Leising et al. 2015). Furthermore, not only did the average monthly SST anomalies reach high temperatures, but the duration of the anomalies was also extended in 2015, with 6 mo being more than 2 standard deviations of the SST recorded for 1988–2015 (Robinson 2016).

Aspects of neonatal physiology that could plausibly be affected by nutrient and energy limitations (Prestes-Carneiro et al. 2006) are erythropoiesis (i.e., red blood cell synthesis) and clearance of damaged erythrocytes (de Back et al. 2014). These processes can be indirectly examined by assessing and quantifying atypical red blood cell morphologies, such as fragmentation, presence of inclusion bodies, and changes in cell size (i.e., anisocytosis), color, shape (i.e., poikilocytosis), or aggregation (i.e., rouleaux; Lynch 1990).

Here we investigated whether anomalies in red blood cell morphology of CSL pups were observable during abnormal SST conditions and whether there was indication that they were related to the severity of the climatic anomaly in the study region (Leising et al. 2015; Robinson 2016). We were particularly interested in determining whether red blood cell alterations could be influenced by pup body condition and by their stable isotope (δ^{13}C and δ^{15}N) values in fur, as a proxy for maternal foraging strategies.

Methods

Study Site and Organism

As part of ongoing, long-term studies on aspects of CSL ecology, during July of 2014 and 2015, we collected blood samples from 49 pups (2014, $n = 22$; 2015, $n = 27$) of 4–6 wk of age that were born at SBA in the Mexican northern Pacific (fig. 1).

CSL pups were captured and manually restrained during the entire procedure, which lasted less than 11 min per pup. Visual inspection by a trained veterinarian determined the general health of each pup and together with the evaluation of leukocyte parameters of the pups (data not shown here) suggested that they were not undergoing any detectable infectious or inflammatory process (Banuet-Martínez et al., forthcoming). The mass of each pup was determined with a vertical hanging scale (max. 60 kg; 0.1 kg precision), and the standard length (tip of the tail to the nose) was measured with a tape measure (1 mm precision). We collected a 2 × 3-cm fur sample from the dorsal region of all but two of the pups captured (2014, $n = 22$; 2015, $n = 25$). Samples were collected using stainless steel scissors that were wiped clean between pups. Fur samples were stored in paper envelopes. A blood sample (5–7 mL) was collected from all pups by puncture of the caudal gluteal vein using a vacuum blood collection tube (Vacutainer, BD Biosciences, Franklin Lakes, NJ) with ethylenediaminetetraacetic acid.

Sampling was conducted by approval of the Bioethics Committee of the Autonomous University of Queretaro. All procedures were carried out under permits SGPA/DGVS/11744/13 and SGPA/DGVS/00195/15 issued by the Dirección General de Vida Silvestre of the Secretaria de Medio Ambiente y Recursos Naturales.

Hematology

Blood was homogenized by inversion of the tubes immediately following collection. Tubes with blood were kept upright inside a cooler and protected from sunlight. We determined the hematocrit (%) by centrifugation of the blood sample in a capillary tube at 11,000 rpm for 5 min. In all cases, this procedure was performed within 8 h of blood collection. We also prepared three blood smears per sample and fixed them in 90% methanol for 10 min. All smears were prepared less than 8 h after the blood was collected. The slides were stained with a Wright solution (Horobin 2011).

To estimate the percentage of anisocytosis (differences in sizes of erythrocytes) and the number of each atypical cell type per pup, we examined the thin-layer zone of the blood smear using a zigzag trajectory and identified areas where erythrocytes were uniformly distributed. We observed 10 fields under light microscopy (×100), and in each we counted each atypical phenotype relative to the number of red blood cells in that field and averaged the percentages across the 10 fields, in this way accounting for interfield differences in cell density.

Data on blood cell morphology of 13 healthy 4- to 6-wk-old CSL pups born in 2012 at Granito Island (see fig. 1), a breeding colony in the Gulf of California, Mexico (A. Flores-Morán, un-

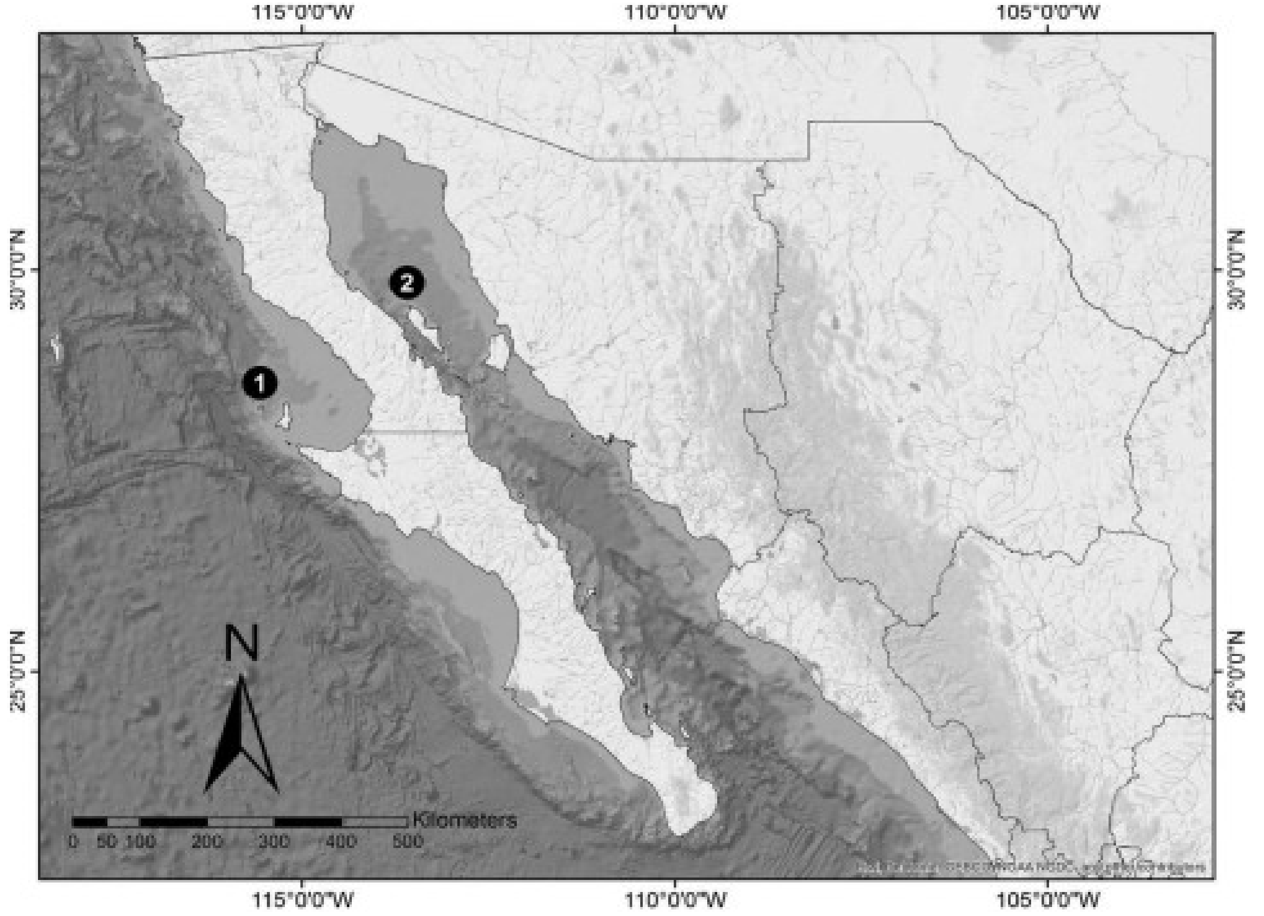

Figure 1. Map that shows the sites at which California sea lion pups were captured and sampled for this study: San Benito Archipelago, in the Mexican northern Pacific (1), and Granito Island, in the Gulf of California (2).

published data), were used as reference values, as these animals were born during a year that was characterized by normal SST (Robinson 2016). Capture, handling, and sampling of those pups and all hematological analyses were conducted exactly as described above.

Isotopic Signatures

We measured values of $\delta^{15}N$ and $\delta^{13}C$ in the fur samples as has been done previously (Elorriaga-Verplancken et al. 2016*b*). Briefly, the fur was cleaned, cut in small pieces, homogenized, and stored in tin capsules before being sent to the University of California at Santa Cruz for analysis in a Carlo Erba 1108 elemental analyzer coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer, with an analytical precision of ±0.2‰ for both stable isotopes. The isotopic compositions were estimated based on known values of standards for each element: Vienna Pee Dee Belemnite for $\delta^{13}C$ and atmospheric for $\delta^{15}N$. We used this approach as pup fur values can act as monitors of their mothers, after a fractionation (consistent isotopic enrichment) between both age groups (Elorriaga-Verplancken et al. 2016*a*), which is similar to a relationship between a predator and its prey (Habran et al. 2010). Because of these consistent differences, the variation between groups of pups is attributed to differences in maternal foraging habits. CSL lanugo is lost gradually around 5 mo of age, and fur starts to grow at around the sixth or seventh month of gestation (Odell 1972). Thus, neonate fur (lanugo) would reflect nutrients that were incorporated starting ~16 wk before collection (~4 wk of lactation and ~12 wk of maternal foraging before giving birth; Odell 1972; Elorriaga-Verplancken et al. 2016*a*). This time frame is similar to that of erythrocyte turnover rates (~12 wk; Kurle 2002). The proximity in these temporal windows facilitated a viable match between the results obtained in the isotopic and the red blood cell analyses, which involved in both cases the final stage of gestation and a first stage of lactation.

Body Mass Index

We calculated the scaled mass index (SMi) for each pup as a measure of body condition. This was done by calculating mass per unit length, taking into account the scaling between body components and body size and applying the equation proposed by Peig and Green (2010):

$$\mathrm{SM}_i = M_i\left[\frac{L_0}{L_i}\right]^{b_{\mathrm{SMA}}},$$

where M_i and L_i were the body mass and standard body length of each pup, respectively; b_{SMA} was the scaling exponent estimated by the regression of mass on body length; and L_0 was the arithmetic mean of the standard body length for each cohort.

Statistical Analysis

We explored our data set graphically to understand the spread and distribution of each parameter. Data that deviated from the normal distribution were further examined with Cullen and Frey graphs to determine their distribution. For each of the atypical erythrocyte morphologies detected, we counted the number of pups in which they were found and built contingency tables to investigate differences in the prevalence between years. For this, we used Pearson's χ^2 tests and determined significance by Fisher exact tests. Differences in the average number of each of the variables between years were examined initially by univariate tests that were chosen according to the distribution of the response variables. Hematocrit values and body condition (SMi) showed a Gaussian distribution, and we used two-tailed t-tests to examine differences between years. In terms of the relative amounts of the aberrant phenotypes, except for microcytes, which showed a Gaussian distribution, all cell types conformed to a beta distribution. We built a series of generalized linear models (GLM; Crawley 2002), and each of the cell types was modeled in turn with a specified error distribution and link (for all except microcytes, a quasibinomial error distribution and logit link were defined). We next built more complex models to examine whether each of the atypical cell types were explained by pup hematocrit (as an indicator of anemia), body condition (SMi), and pup isotopic signal (δ^{15}N and δ^{13}C). All analyses were conducted in R, version 3.3.1 (R Development Core Team 2016), using core packages as well as epitools, descdist, and ggplot2.

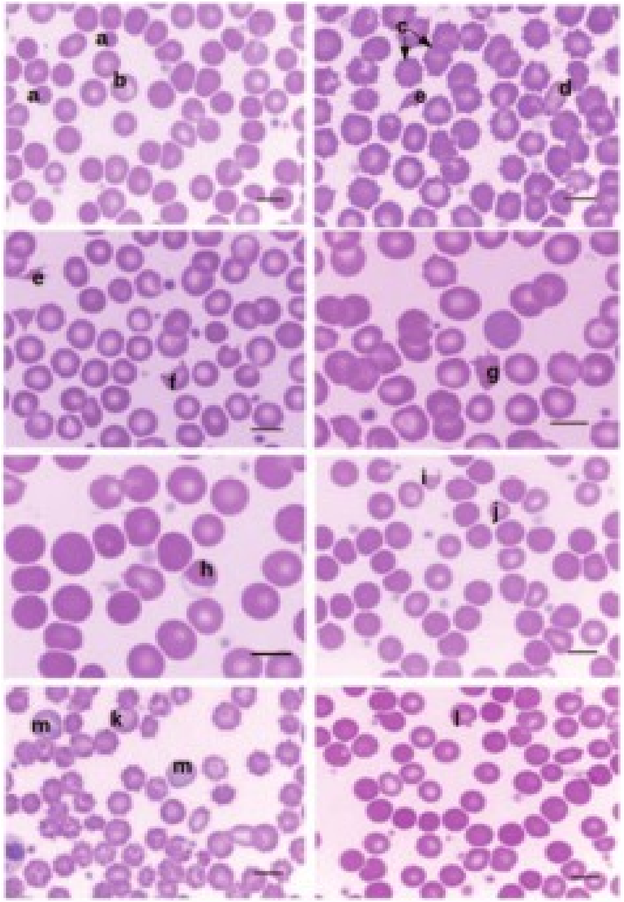

Figure 2. Atypical red blood cells observed in blood smears from California seal lion pups born at San Benito Archipelago during abnormal sea surface temperature conditions. *a*, Spherocyte. *b*, Codocyte (target cell). *c*, Echinocyte (burr cell). *d*, Elliptocyte. *e*, Schistocyte. *f*, Keratocyte. *g*, Acantocytes (spur cells). *h*, Eccentrocyte. *i*, Dacrocyte. *j*, Microcyte. *k*, Macrocyte. *l*, Heinz body. *m*, Polychromatophilic RBC (reticulocyte). Wright stain; ×100. Scale bar = 10 μm.

Results

Atypical erythrocyte morphologies were observed in all of the blood smears from pups born in 2014 and 2015. Specifically, we detected reticulocytes (polychromatophilic red blood cells), microcytes, macrocytes, acanthocytes (spur cells), codocytes (target cells), dacrocytes, eccentrocytes, echinocytes (burr cells), elliptocytes, keratocytes, schistocytes, spherocytes, and Heinz bodies (fig. 2). Rouleaux was not observed in any of the blood smears. Several of the atypical cell types (reticulocytes, acanthocytes, eccentrocytes, echinocytes, elliptocytes, keratocytes, and spherocytes) were significantly more prevalent in pups born during high SST years than in apparently healthy CSL pups born in 2012 ($P < 0.05$ in all tests), and only codocytes were more prevalent in pups born in 2012 than in either of the high SST years (χ^2 tests, $P < 0.01$). Only four of the detected phenotypes varied significantly in prevalence between 2014 and 2015; namely, fewer pups born in 2014 had codocytes ($\chi^2 = 14.73$, $P = 0.001$) and keratocytes ($\chi^2 = 8.59$, $P = 0.003$) than those born in 2015, while the inverse pattern was seen for reticulocytes ($\chi^2 = 14.75$, $P = 0.0001$) and elliptocytes ($\chi^2 = 4.36$, $P = 0.029$). The prevalence of each of these atypical cell morphologies per sampling cohort is shown in table 1.

When investigating interyear differences in the counts of each atypical cell type, we found that nearly all were significantly higher in pups born during the abnormally high SST years than in those born in 2012. The amount of dacrocytes, schistocytes, and Heinz bodies per smear of pups born during the atypical SST years were similar to those of pups born in 2012. With the exception of codocyte counts, which were lower in pups born during the atypical SST years (2014 vs. 2012; GLM, $F_{1,33} = 44.58$, $P = 1.33 \times 10^{-07}$; 2014 vs. 2012; GLM, $F_{1,37} = 28.09$, $P = 5.57 \times 10^{-06}$), all other atypical cells were more abundant

Table 1: Prevalence of atypical erythrocyte morphologies detected in California sea lion pup blood smears

Morphology	2014; $n = 22$	2015; $n = 27$	2012; $n = 13$
Anisocytosis	100	96.30	100
Reticulocytes	**77.27**	**22.22**	61.53[b]
Microcytes	100	92.59	100
Macrocytes	100	92.59	92.31
Acanthocytes	59.09	81.48	23.08[a,b]
Codocytes	**0**	**37.04**	84.62[a,b]
Dacrocytes	59.09	62.96	69.23
Eccentrocytes	54.55	59.26	0[a,b]
Echinocytes	77.27	96.30	30.77[a,b]
Elliptocytes	**86.36**	**59.26**	46.15[a]
Keratocytes	**4.55**	**40.74**	46.15[a]
Schistocytes	86.36	100	100
Spherocytes	95.45	100	53.85[a,b]
Heinz bodies	0	7.41	0

Note. Columns show the prevalence recorded for pups born during the 2014 and 2015 sea surface temperature (SST) anomalies. The final column shows the prevalence of each of the atypical red blood cell morphologies in pups of the same age born in 2012, when the SST was normal. Values in boldface differed significantly between years.

[a] Significant differences between 2014 and 2012.

[b] Significant differences between 2015 and 2012.

than those recorded for pups born in 2012 ($P < 0.01$ for all models). Reticulocytes, microcytes, and elliptocytes were more abundant in the blood of pups born in 2014 than in 2015, while codocytes and keratocytes showed the inverse trend, being more abundant in pups born in 2015 ($P < 0.01$ for all models; see table 2 for average values and range of each sampling cohort).

CSL pup hematocrit values remained equal between years (2014, mean = 44.75%, SD = 4.82%; 2015, mean = 47.94%, SD = 5.06%; t-test, $t = -1.974$, df = 33.02, $P = 0.057$) and did not differ from that recorded for apparently healthy CSL pups born at Granito in 2012, when SST was normal (hematocrit mean = 46.24%, SD = 11.79%; t-tests: $t = -0.101$, df = 7.48, $P = 0.923$). Of all the atypical erythrocyte morphologies observed in pups born at SBA, only microcytes were inversely related to pup hematocrit (GLM; $F_{1,39} = 10.40$, $P = 0.003$, adj. $R^2 = 0.19$; fig. 3*A*), regardless of the year of birth.

Body condition was significantly lower for pups born in 2015 than in 2014, when the SST conditions were milder (two-tailed t-test = 2.152, df = 44.91, $P = 0.04$). The number of microcytes was directly related to pup body condition, regardless of year of birth (GLM; $F_{1,46} = 6.52$, $P = 0.015$). None of the other red blood cell phenotypes were influenced by pup body condition ($P > 0.05$ for all models).

Isotopic values of fur differed significantly between 2014 and 2015 (δ^{13}C: $t = 3.8$, $P = 0.00001$; δ^{15}N: $t = -5.9$, $P = 0.0003$). In 2014, mean (±SD) CSL pup values were −16.2‰ ± 0.4‰ (δ^{13}C) and 19.8‰ ± 0.4‰ (δ^{15}N). In 2015, the mean (±SD) values were −16.9‰ ± 0.4‰ (δ^{13}C) and 20.2‰ ± 0.4‰ (δ^{15}N). There was no significant relationship between either of the stable isotopes and pup hematocrit ($P > 0.05$ for both models). However, pups with higher δ^{13}C values tended to have higher numbers of microcytes (GLM; $F_{1,45} = 7.97$, $P = 0.007$, adj. $R^2 = 0.10$; fig. 3*B*) and reticulocytes (GLM; $F_{1,45} = 30.63$, $P = 1.71 \times 10^{-06}$; fig. 3*C*). Values for δ^{15}N were inversely related to the amount of microcytes (GLM; $F_{1,45} = 13.44$, $P = 0.001$;

Table 2: Relative amount of anisocytosis and atypical erythrocyte morphologies in California sea lion pup blood smears

Morphology	2014 median (%; range); $n = 22$	2015 median (%; range); $n = 27$	2012 median (%; range); $n = 13$
Anisocytosis	9.31 (2.2–16.30)	8.11 (0–20.30)	4.89[a,b] (1.02–17.13)
Reticulocytes	**2.15 (0–18.27)**	**.26 (0–2.83)**	.15[a] (0–1.17)
Microcytes	**9.02 (2.12–15.85)**	**4.97 (0–9.85)**	.31[a,b] (0–.82)
Macrocytes	3.51 (.46–8.43)	3.98 (0–10.18)	1.50[a,b] (0–3.89)
Acanthocytes	2.62 (0–27.57)	7.05 (0–47.25)	.59[b] (0–2.83)
Codocytes	**0 (0–0)**	**.12 (0–.54)**	1.83[a,b] (0–8.44)
Dacrocytes	.56 (0–2.93)	.45 (0–2.14)	.15 (0–.42)
Eccentrocytes	.68 (0–2.28)	.70 (0–2.51)	0[a,b] (0–0)
Echinocytes	19.65 (0–80.09)	23.08 (0–50.37)	7.73[b] (0–36.80)
Elliptocytes	**.89 (0–2.71)**	**.30 (0–1.22)**	.05[a,b] (0–.16)
Keratocytes	**.01 (0–.13)**	**.11 (0–.77)**	.05[b] (0–.16)
Schistocytes	.42 (0–2.17)	.56 (.02–1.41)	.43 (.13–.91)
Spherocytes	24.07 (0–82.20)	14.43 (2.54–46.58)	.38[a,b] (0–1.55)
Heinz bodies	0 (0–0)	.02 (0–.32)	0 (0–0)

Note. Columns show the median and range observed for pups born during the 2014 and 2015 sea surface temperature (SST) anomalies. Values in boldface differed significantly between 2014 and 2015. The final column shows the mean and range of each of the atypical red blood cell morphologies detected in pups of the same age born in 2012, when the SST was normal.

[a] Significant differences between 2014 and 2012.

[b] Significant differences between 2015 and 2012.

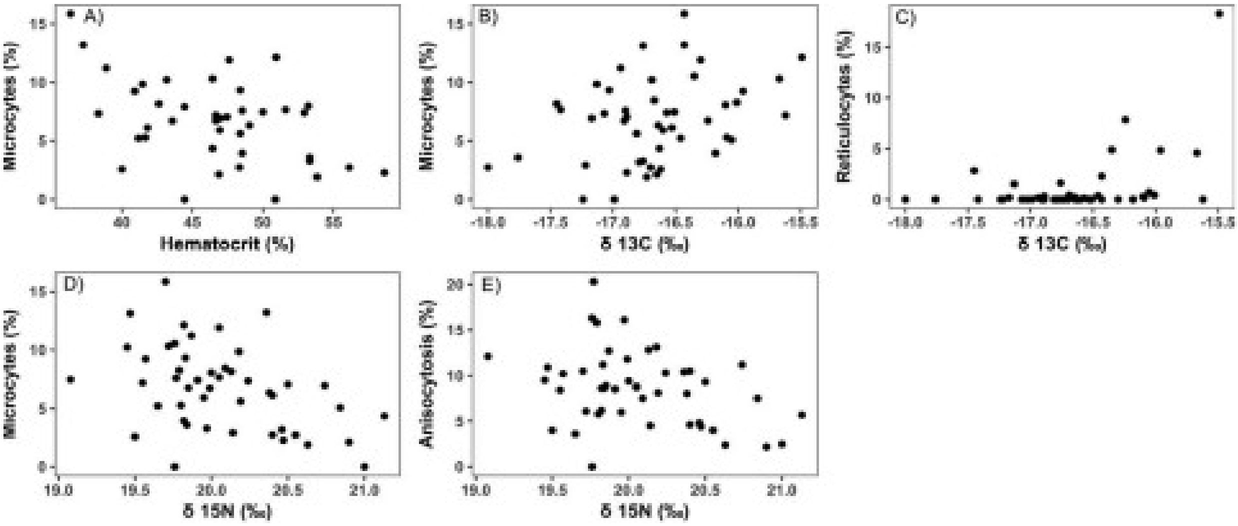

Figure 3. Variation in the number of atypical erythrocyte morphologies in terms of hematocrit (%) and fur isotopic signal (‰). *A*, Relationship between hematocrit and microcytes. *B*, Isotopic δ^{13}C values and microcytes. *C*, Isotopic δ^{13}C values and reticulocytes. *D*, Isotopic δ^{15}N values and microcytes. *E*, Isotopic δ^{15}N values and anisocytosis.

fig. 3*D*) and to anisocytosis (GLM; $F_{1,45} = 5.74$, $P = 0.021$; fig. 3*E*).

Discussion

Red blood cells are indispensable to vertebrates as they bind oxygen and deliver it to tissues and organs. In order to function adequately, the cells rely on membrane stability and hemoglobin concentration (McGuire and Agre 1988; Lux 2016). If these are altered due to inherited, congenital, infectious, or autoimmune diseases, trauma, or nutritional deficiencies, their physiology is impaired and the organism's health can be seriously compromised (Naigamwalla et al. 2012). Here we have characterized red blood cell morphology of California sea lion pups that were born during 2014 and 2015 on the San Benito Archipelago, in the Mexican north Pacific, when the anomalously high SST event known as the Blob (Bond et al. 2015; Kintisch 2015) and a simultaneous El Niño event had reached this geographic region (Robinson 2016), posing a challenge for pregnant and lactating CSL females that increased their feeding effort in order to procure the dietary requirements needed for their maintenance and that of their pup (Elorriaga-Verplancken et al. 2016*b*).

We found a number of atypical erythrocyte types in the blood of the CSL pups. Regrettably, data on normal erythrocyte morphology of neonatal CSL are unavailable. Most studies on free-ranging otariid neonates published to date have reported total red blood cell (RBC) counts and hematocrit and hemoglobin values, and (less often) mean cell volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin (e.g., Needham et al. 1980; Castellini et al. 1993; Horning and Trillmich 1997; Beckmen et al. 2003; Brock et al. 2013). To the best of our knowledge, only one other study has evaluated erythrocyte morphology of otariid pinnipeds (Clark et al. 2002) and reported a range of cells with nontypical morphology, including echinocytes and schistocytes, as observed here. However, in most individuals of the five Australasian otariid species studied (Clark et al. 2002), these cells were found in lower numbers than those we recorded for the CSL pups and reticulocytes were uncommon (Clark et al. 2002), while we found them in 22% (2015) and 77% (2014) of the pups. Furthermore, the aforementioned study included wild and captive individuals and did not provide information on the individuals' age. This is relevant, as some altered morphologies can occur during early developmental stages. For instance, fetal erythrocytes tend to be larger and can vary more in shape than those of adult humans (Zipurksy et al. 1983; Steiner and Gallagher 2007), and peripheral blood smears of infants have greater numbers of acanthocytes, target cells, and reticulocytes than adults (Zipurksy et al. 1983), presumably due to decreased deformability and increased osmotic resistance relative to adult's RBC (Christensen 2000).

Based on the above, it could be argued that at least some of the unusual cell morphologies observed in the CSL pup blood smears of our study could be normal for their stage of development. This could be the case for schistocytes, dacrocytes, and Heinz bodies whose prevalence and abundance per smear were statistically similar to those recorded for pups of the same age that were born at Granito Island in 2012, when SST was normal. That being said, blood smears of the CSL pups born during high SST years revealed some marked differences to those of pups born in 2012. First, none of the latter had eccentrocytes, which were detected in more than half of the pups born during the high SST years, suggesting that their presence in pups is unlikely to be normal. The average counts of codocytes were lower in 2014 and 2015 than in 2012, although all values were within the reference range, making this finding unlikely to be of biological significance. Furthermore, except for schistocytes and Heinz bodies, all abnormalities that were present in the blood of pups born during the high SST years were significantly more abundant than in pups born during 2012, plausibly implying that their presence could indicate physiological alterations, which we will discuss below.

In general, erythrocyte aberrations are formed due to membrane abnormalities of congenital or nutritional etiology or are caused by intravascular trauma (Naigamwalla et al. 2012; Lux 2016). The fact that the CSL pups included in this study showed no signs of trauma or infectious disease that could explain damage to the erythrocytes led us to propose that their occurrence might be due to suboptimal nutrition (see Billett 1990; Koury 2014) derived from increased maternal foraging effort (Kuhn and Costa 2014; Elorriaga-Verplancken et al. 2016*b*) and reduced dietary quality of prey consumed (McClatchie et al. 2016) during the high SST events that occurred in the northeastern Pacific during the study years. In particular, reticulocytes, microcytes, spherocytes, and echinocytes—all detected in higher numbers in pups born during the high SST years—tend to be formed when an individual's uptake of iron is inadequate (England et al. 1976; Clark 2009; Collard 2009) or when there is altered intestinal absorption of iron, folic acid, and vitamin B12 (Lipinski et al. 2010).

While tempting to discuss the abnormal phenotypes as unequivocal evidence of nutritional anemia, having found that the pups' hematocrit did not differ from that of the reference values makes it somewhat challenging to support this argument. One possibility is that if females spent less time nursing their pups during the high SST years, consumption of liquids and nutrients by the pup would be suboptimal and their plasma volume would decrease, leading their hematocrit to appear higher than if they were normovolemic, as occurs during dehydration (Billet 1990; Erslev 1997). Also, it is possible that at the time of sampling, any potential anemic state had already been resolved, and had the pups been sampled earlier, we might have found low hematocrit values. In other words, our results are a snapshot of a specific time period, and we can only speculate about what occurred previously. Nonetheless, taking into account that the prevalence and abundance of specific cell types varied between 2014 and 2015, we propose that distinct processes were occurring among those years.

Namely, reticulocytes, microcytes, and elliptocytes were more abundant in 2014, while codocytes and keratocytes were more abundant in 2015. Both microcytes and keratocytes are indicative of nutrient deficiency (Massey 1992; Naigamwalla et al. 2012; Thrall et al. 2012), while reticulocytes occur during active erythropoiesis (Janus and Moerskel 2010). Thus, we propose that pups born in 2014 were undergoing active erythropoiesis as a medullary response to hypoxia (Haase 2010). Such hypoxia could be the result of developmental demands (Halvorsen and Bechensteen 2002) or could be the clinical footprint of an already resolved anemic state (Lux 2016). In contrast, pups born in 2015 had cell types that tend to be associated with iron deficiencies and less evidence of active (responsive) erythropoiesis than pups born in 2014. This would suggest that the medullary response was less adequate in pups born during the most severe SST anomaly.

Pinniped milk composition has yet to be studied beyond lipids, proteins, carbohydrates, sodium, and potassium (e.g., Dosako et al. 1983; Trillmich et al. 1988; Arnould and Hindell 1999; Georges et al. 2001; Cane et al. 2005), and we are unaware of any published study that has examined and quantified the presence of these elements in the milk of any pinniped species. However, traces of iron, folate, and various other micronutrients are components of human milk (Bullen et al. 1972; Allen 2012) and that of various other mammals, including dogs (Adkins et al. 2001), cats (Adkins et al. 1997), and giant pandas (Zhang et al. 2016). In fact, there appear to be marked commonalities in milk composition among mammalian species (Davis et al. 1995; but see Sharp et al. 2006), so it is likely that CSL pups normally receive these micronutrients during lactation. While we do not know whether the composition of CSL milk would vary with climatic alterations, based on the observed relationship between the isotopic signal and the numbers of some of the aberrant RBC morphologies, it would appear that changes in maternal foraging habits during high SST events could have an impact on their pup's micronutrient intake. Even if milk composition remained unchanged despite adult females feeding on prey of less nutritional value (Sharp et al. 2006) or performing extended foraging trips due to the anomalously high SST (McClatchie et al. 2016), lactating pups would presumably receive fewer nutrients, including those essential for adequate erythropoiesis. For neonates, this could be problematic, as during periods of rapid growth, even more iron is needed (Lipinski et al. 2010).

If, as hypothesized, maternal nutritional limitations driven by the abnormal SST conditions lead to suboptimal nutrition of the pups, and given that the environmental conditions that challenged adult females were less severe in 2014 than in 2015 in the study region (Robinson 2016), presumably pups born in 2014 would have received enough nutrients to be able to compensate for transient hypoxia due to active erythropoiesis (Halvorsen and Bechensteen 2002; Haase 2010) or iron depletion (Massey 1992; Janus and Moerskel 2010; Naigamwalla et al. 2012; Thrall et al. 2012). Having found that lower fur $\delta^{13}C$ values were associated with fewer numbers of reticulocytes and microcytes provides some evidence for this hypothesis. More negative $\delta^{13}C$ values indicate more oceanic feeding (Hobson 1990; Burton and Koch 1999) typical of an extended foraging range of adult females in search of prey (Boness and Bowen 1996; Elorriaga-Verplancken et al. 2016*a*). It is possible that the extended lapses without suckling hinder the pups' mechanisms to meet physiological demands or nutritional alterations. It was interesting to find no evidence that body condition was related to the number of red blood cells that indicate active erythropoiesis, suggesting that pups could have a relatively high mass-to-length ratio without necessarily possessing sufficient nutrients to implement biochemical and cellular processes adequately, as can occur in humans (Abdullah 2015).

The abundance of microcytes and anisocytosis were inversely related to fur $\delta^{15}N$. Intense fasting can cause a negative nitrogen balance and a ^{15}N-enriched nitrogen pool, which is involved in amino acid synthesis (Hobson et al. 1993). However, we did not find evidence of emaciation or serious malnutrition in any of the sampled pups that would suggest this scenario. An alternative explanation is that the higher δ^{15}N values are indicative of pregnant or nursing mothers feeding on prey of higher trophic position (see Minagawa and Wada 1984). It was recently reported that adult CSL off the California coast had switched to prey of a higher trophic level (rock fishes) than their regular prey (sardines and anchovies) in this region, presumably due to the fact that the high SST caused by the Blob diminished prey availability (McClatchie et al. 2016). Taking into consideration that microcytes and reticulocytes were less abundant within each blood smear in 2015 than in 2014, it is possible that CSL pups suckling from adult females that fed on less nutritious prey of a higher trophic position were less able to compensate for any micronutrient deficiency. The proximity in the turnover of fur (Odell 1972) and erythrocytes (Kurle 2002) facilitated a viable match between the results obtained in the isotopic and RBC analyses, which involved in both cases the final stage of gestation and a first stage of lactation (Odell 1972; Elorriaga-Verplancken et al. 2016*a*).

In addition to micronutrient restriction and active erythropoiesis, another (nonexclusive) possibility is that at least some of the atypical morphologies observed herein reflect suboptimal phagocytosis by macrophages. These phagocytic cells play an important role in erythropoiesis and in clearance of old or defective blood cells. Macrophages found in the bone marrow provide iron for developing erythroblasts (Bessis and Breton-Gorius 1962; Leimberg et al. 2008; Hentze et al. 2010), while those in the liver and spleen undertake clearance of old or damaged erythrocytes and are thus responsible for providing iron to the bone marrow from recycling of destroyed erythrocytes (de Back et al. 2014). If macrophage physiology were altered due to dietary energy constraints, both erythropoiesis and erythrocyte destruction could plausibly be impaired, leading to the accumulation of abnormal cells. This could occur without necessarily altering the number of circulating monocytes. We have no means of addressing this hypothesis experimentally; however, impaired immune activity, including responses reliant on phagocytosis (see Salaberria et al. 2013), have been observed in CSL pups born under these anomalous SST conditions, despite normal leukocyte counts (Banuet-Martinez et al., forthcoming), so it is possible that the phagocytic capability of hepatic and splenic macrophages was impaired in CSL pups born to mothers under nutritional duress.

If any of the proposed scenarios were proven correct, the presence of atypical red blood cells could be relevant to CSL pup fitness, as red blood cells are particularly important for developing individuals that will rely heavily on increased oxygen storage capacity for diving and foraging (Clark et al. 2006; Weise and Costa 2007; Burns et al. 2015). In the current context of sudden, striking, and often unpredictable environmental changes, such as the Blob and other potential climatic events, it is imperative to increase our understanding of the effects that they can have on natural populations, particularly in terms of the long-term consequences that could affect species with slow sexual maturity and low reproductive rates. This study is a first step to explore the effects that abnormally high SST can have on key physiological aspects of developing individuals.

Acknowledgments

We thank L. Soto-Garcia and C. Barragán-Vargas for their help during pup captures and sample processing in the field and the Abalone Fishermen Cooperative (Pescadores Nacionales de Abulón) from Cedros Island for logistical support. E. Vargas Perusquia greatly assisted with grant administration. Author contributions are as follows: K.A.-W. conceived and designed the study; M.B.-M., A.F.-M., and J.S.-S. processed the samples and collected the data; K.A.-W., F.R.E.-V., and L.E.G.-O. analyzed the data; K.A.-W. and A.F.-M. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. This study was supported by a grant from the Fund for the Advancement of Scientific Research (grant FNB201602). A.F.-M. and M.B.-M. are funded by CONACYT graduate scholarships (grants 287579 and 778607).

Literature Cited

Abdullah A. 2015. The double burden of undernutrition and overnutrition in developing countries: an update. Curr Obes Rep 4:337–349.

Adkins Y., A.J. Lepine, and B. Lonnerdal. 2001. Changes in protein and nutrient composition of milk throughout lactation in dogs. Am J Vet Res 62:1266–1272.

Adkins Y., S.C. Zicker, A. Lepine, and B. Lonnerdal. 1997. Changes in nutrient and protein composition of cat milk during lactation. Am J Vet Res 58:370–375.

Allen L.H. 2012. Vitamin B-12. Adv Nutr 3:54–55.

Arnould J.P. and M.A. Hindell. 1999. The composition of Australian fur seal (*Arctocephalus pusillus doriferus*) milk throughout lactation. Physiol Biochem Zool 72:605–612.

Banuet-Martinez M., W. Espinosa-de Aquino, F.R. Elorriaga-Verplancken, A. Flores-Morán, O.P. Garcia, M. Camacho, and K. Acevedo-Whitehouse. Forthcoming. Climatic anomaly affects body condition, energetic resources, and immune competence of a marine top predator. PLoS ONE.

Beckmen K.B., J.E. Blake, G.M. Ylitalo, J.L. Stott, and T.M. O'Hara. 2003. Organochlorine contaminant exposure and associations with hematological and humoral immune functional assays with dam age as a factor in free-ranging northern fur seal pups (*Callorhinus ursinus*). Mar Poll Bull 46:594–606.

Bessis M.C. and J. Breton-Gorius. 1962. Iron metabolism in the bone marrow as seen by electron microscopy: a critical review. Blood 19:635–663.

Billett H.H. 1990. Hemoglobin and haematocrit. Pp. 718–719 in H.K. Walker, W.D. Hall, and J.W. Hurst, eds. Clinical methods: the history, physical, and laboratory examination. Butterworths, Boston.

Bond N.A., M.F. Cronin, H. Freeland, and N. Mantua. 2015. Causes and impacts of the 2014 warm anomaly in the NE Pacific: 2014. Geophys Res Lett 42:3414–3420.

Boness D.J. and W.D. Bowen. 1996. Evolution of maternal care in pinnipeds. BioSci 46:645–654.

Boness D.J., O.T. Oftedal, and K.A. Ono. 1991. The effect of El Niño on pup development in the California sea lion (*Zalophus californianus*) early postnatal growth. Pp. 173–184 in F. Trillmich and K. Ono, eds. Pinnipeds and El Niño: responses to environmental stress. Springer, Berlin.

Brock P., A. Hall, S. Goodman, M. Cruz, and K. Acevedo-Whitehouse. 2013. Applying the tools of ecological immunology to conservation: a test case in the Galapagos sea lion. Anim Conserv 16:19–31.

Bullen J.J., H.R. Rogers, and L. Leigh. 1972. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. Br Med J 1:69–75.

Burns J.M., K. Lestyk, D. Freistroffer, and M.O. Hammill. 2015. Preparing muscles for diving: age-related changes in muscle metabolic profiles in harp (*Pagophilus groenlandicus*) and hooded (*Cystophora cristata*) seals. Physiol Biochem Zool 88:167–182.

Burton R.K. and P.L. Koch. 1999. Isotopic tracking of foraging and long distance migration in northeastern Pacific pinnipeds. Oecologia 119:578–585.

Cane K.N., J.P. Arnould, and K.R. Nicholas. 2005. Characterisation of proteins in the milk of fur seals. Comp Biochem Physiol B Biochem Mol Biol 141:111–120.

Castellini M.A., R.W. Davis, T.R. Loughlin, and T.M. Williams. 1993. Blood chemistries and body condition of Steller sea lion pups at Marmot Island, Alaska. Mar Mamm Sci 9: 202–208.

Christensen R.D. 2000. Expected hematologic values for term and preterm neonates. Pp. 117–136 in R.D. Christensen, ed. Hematologic problems of the neonate. Saunders, Philadelphia.

Chust G., J.I. Allen, L. Bopp, C. Schrum, J. Holt, K. Tsiaras, M. Zavatarelli, et al. 2014. Biomass changes and trophic amplification of plankton in a warmer ocean. Global Change Biol 20:2124–2139.

Clark C.A., J.M. Burns, J.F. Schreer, and M.O. Hammill. 2006. Erythropoietin concentration in developing harbor seals (*Phoca vitulina*). Gen Comp Endocrinol 147:262–267.

Clark P., W.S. Boardman, and P.J. Duignan. 2002. Cytology of haematological cells of otariid seals indigenous to Australasian waters. Aust Vet J 80:161–164.

Clark S.F. 2009. Iron deficiency anemia: diagnosis and management. Curr Opin Gastroenterol 25:122–128.

Collard K.J. 2009. Iron homeostasis in the neonate. Pediatrics 123:1208–1216.

Crawley M.J. 2002. Statistical computing, an introduction to data analysis using S-plus. J. Wiley, Chichester.

Davis T.A., H.V. Nguyen, D.P. Costa, and P.J. Reeds. 1995. Amino acid composition of pinniped milk. Comp Biochem Physiol B Biochem Mol Biol 110:633–639.

de Back D.Z., E.B. Kostova, M. van Kraaij, T.K. van den Berg, and R. van Bruggen. 2014. Of macrophages and red blood cells: a complex love story. Front Physiol 5:9.

Dosako S., S. Taneya, T. Kimura, T. Ohmori, H. Daikoku, N. Suzuki, J. Sawa, K. Kano, and S. Katayama. 1983. Milk of northern fur seal: composition, especially carbohydrate and protein. J Dairy Sci 66:2076–2083.

Elorriaga-Verplancken F.R., A. Juárez-Ruiz, M. Aquino-Baleytó, F. Galván-Magaña, and S. Aguíñiga-García. 2016*a*. Isotopic variation between adult female Guadalupe fur seals and their offspring: implications for the use of neonates as proxies for maternal foraging. Aquat Mammals 42:268–276.

Elorriaga-Verplancken F.R., G.E. Sierra-Rodriguez, H. Rosales-Nanduca, K. Acevedo-Whitehouse, and J. Sandoval-Sierra. 2016*b*. Impact of the 2015 El Niño-Southern Oscillation on the abundance and foraging habits of Guadalupe fur seals and California sea lions from the San Benito Archipelago, Mexico. PLoS ONE e0155034.

England J.M., S.M. Ward, and M.C. Down. 1976. Microcytosis, anisocytosis and the red cell indices in iron deficiency. Br J Haematol 34:589–597.

Erslev A.J. 1997. Clinical erythrokinetics: a critical review. Blood Rev 11:160–167.

France R.L. 1995. Carbon-13 enrichment in benthic compared to planktonic algae: foodweb implications. Mar Ecol Prog Ser 124:307–312.

García-Rodríguez F.J. and D. Aurioles-Gamboa. 2004. Spatial and temporal variation in the diet of the California sea lion (*Zalophus californianus*) in the Gulf of California, Mexico. Fish Bull 102:47–62.

Georges J.-Y., R. Groscolas, C. Guinet, and J.P. Robin. 2001. Milking strategy in subantarctic fur seals *Arctocephalus tropicalis* breeding on Amsterdam Island: evidence from changes in milk composition. Physiol Biochem Zool 74:548–559.

Haase V.H. 2010. Hypoxic regulation of erythropoiesis and iron metabolism. Renal Physiol 299:1–13.

Habran S., C. Debier, D.E. Crocker, D.S. Houser, G. Lepoint, J.M. Bouquegneau, and K. Das. 2010. Assessment of gestation, lactation and fasting on stable isotope ratios in northern elephant seals (*Mirounga angustirostris*). Mar Mammal Sci 26:880–895.

Halvorsen S. and A.G. Bechensteen. 2002. Physiology of erythropoietin during mammalian development. Acta Paediatr Suppl 91:17–26.

Hentze M.W., M.U. Muckenthaler, B. Galy, and C. Camaschella. 2010. Two to tango: regulation of mammalian iron metabolism. Cell 142:24–38.

Hobson K.A. 1990. Stable isotope analysis of marine bled murrelets: evidence for freshwater feeding and determination of trophic level. Condor 92:897–903.

Hobson K.A., R.T. Alisauskas, and R.G. Clark. 1993. Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analysis of diet. Condor 95:388–394.

Horning M. and F. Trillmich. 1997. Development of hemoglobin, hematocrit, and erythrocyte values in Galápagos fur seals. Mar Mamm Sci 13:100–113.

Horobin R. 2011. How Romanowsky stains work and why they remain valuable—including a proposed universal Romanowsky staining mechanism and a rational troubleshooting scheme. Biotech Histochem 86:36–51.

Janus J. and S.K. Moerschel. 2010. Evaluation of anemia in children. Am Fam Physician 81:1462–1471.

Kintisch E. 2015. "The Blob" invades Pacific, flummoxing climate experts. Science 348:17–18.

Koury M.J. 2014. Abnormal erythropoiesis and the pathophysiology of chronic anemia. Blood Rev 28:49–66.

Kuhn C.E. and D.P. Costa. 2014. Interannual variation in the at-sea behavior of California sea lions (*Zalophus californianus*). Mar Mamm Sci 30:1297–1319.

Kurle C.M. 2002. Stable-isotope ratios of blood components from captive northern fur seals (*Callorhinus ursinus*) and their diet: applications for studying the foraging ecology of wild otariids. Can J Zool 80:902–909.

Leimberg M.J., E. Prus, A.M. Konijn, and E. Fibach. 2008. Macrophages function as a ferritin iron source for cultured human erythroid precursors. J Cell Biochem 103:1211–1218.

Leising A.W., I.D. Schroeder, S.J. Bograd, J. Abell, R. Durazo, R.G. Gaxiola-Castro, E.P. Bjorkstedt, et al. 2015. State of the California Current 2014–15: impacts of the warm water "Blob." Calif Coop Fish Invest Rep 56:31–68.

Lipinski P., R.R. Starzynski, F. Canonne-Hergaux, B. Tudek, R. Olinski, P. Kowalczyk, T. Dziaman, et al. 2010. Benefits and risks of iron supplementation in anemic neonatal pigs. Am J Pathol 177:1233–1243.

Lowry M.S., B.S. Stewart, C.B. Heath, P.K. Yochem, and J.M. Francis. 1990. Seasonal and annual variability in the diet of California sea lions *Zalophus californianus* at San Nicolas Island, California, 1981–86. Fish Bull 89:331–336.

Lux S.E., 4th. 2016. Anatomy of the red cell membrane skeleton: unanswered questions. Blood 127:187–199.

Lynch E.C. 1990. Peripheral blood smear. Pp. 732–734 in H.K. Walker, W.D. Hall, and J.W. Hurst, eds. Clinical methods: the history, physical, and laboratory examinations. Butterworths, Boston.

Massey A.C. 1992. Microcytic anemia: differential diagnosis and management of iron deficiency anemia. Med Clin North Am 76:549–566.

McClatchie S., J. Field, A.R. Thompson, T. Gerrodette, M. Lowry, P.C. Fiedler, W. Watson, K.M. Nieto, and R.D. Vetter. 2016. Food limitation of sea lion pups and the decline of forage off central and Southern California. Roy Soc Open Sci, doi:10.1098/rsos.150628.

McGuire M. and P. Agre. 1988. Clinical disorders of the erythrocyte membrane skeleton. Hematol Pathol 2:1–14.

Melin S.R., A.J. Orr, J.D. Harris, J.I. Laakei, F.M.D. Gulland, and S. Stoudt. 2010. Unprecedented mortality of California sea lion pups associated with anomalous oceanographic conditions along the central California coast in 2009. CalCOFI Rep 51:182–194.

Michener R.H. and D.M. Schell. 1994. Stable isotope ratios as tracers in marine aquatic food webs. Pp. 138–157 in K. Lajtha and R.H. Michener, eds. Stable isotopes in ecology. Blackwell Scientific, New York.

Minagawa M. and E. Wada. 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between δ^{15}N and animal age. Geochim Cosmochim Acta 48:1135–1140.

Naigamwalla D.Z., J.A. Webb, and U. Giger. 2012. Iron deficiency anemia. Can Vet J 53:250–256.

Needham D.J., C.J. Cargill, and D. Sheriff. 1980. Haematology of the Australian sea lion, *Neophoca cinerea*. J Wildl Dis 16:103–108.

Odell D.K. 1972. Studies on the biology of the California sea lion and the northern elephant seal on San Nicolas Island. PhD diss. University of California, Los Angeles.

Ono K.A., D.J. Boness, O.T. Oftedal, and S.J. Iverson. 1993. The effects of El Nino on mother-pup biology in the California sea lion. Pp 495–500. In F.G. Hochberg, ed. Recent advances in research on the California islands. Santa Barbara Museum of Natural History, Santa Barbara, CA.

Peig J. and A.J. Green. 2010. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118:1883–1891.

Prestes-Carneiro L.E., R.D. Laraya, P.R.C. Silva, R.A. Moliterno, I. Felipe, and P.C. Mathias. 2006. Long-term effect of early protein malnutrition and growth curve, hematological parameters and macrophage function of rats. J Nutr Sci Vitaminol 52:414–420.

R Development Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.r-project.org.

Robinson C.J. 2016. Evolution of the 2014–2015 sea surface temperature warming in the central west coast of Baja California, Mexico, recorded by remote sensing. Geophys Res Lett 43:7066–7071.

Salaberria C., J. Muriel, M. de Luna, D. Gil, and M. Puerta. 2013. The PHA test as an indicator of phagocytic activity in a passerine bird. PLoS ONE e84108.

Sharp J.A., K.N. Cane, C. Lefevre, J.P. Arnould, and K.R. Nicholas. 2006. Fur seal adaptations to lactation: insights into mammary gland function. Curr Top Develop Biol 72:275–308.

Stearns S.C. 1992. The evolution of life histories. Oxford University Press, Oxford.

Steiner L.A. and P.G. Gallagher. 2007. Erythrocyte disorders in the perinatal period in adverse pregnancy outcome and the fetus/neonate. Semin Perinatol 31:254–261.

Thrall M.A. 2012. Erythrocyte morphology. Pp 61–74 in M.A. Thrall, G. Weiser, R. Allison and T.W. Campbell, eds. Veterinary hematology and clinical chemistry. Wiley-Blackwell, New York.

Trillmich F., D. Kirchmeier, O. Kirchmeier, I. Krause, E. Lechner, H. Scherz, H. Eichinger, and M. Seewald. 1988. Characterization of proteins and fatty acid composition in Galapagos fur seal milk. Occurrence of whey and casein protein polymorphisms. Comp Biochem Physiol B Biochem Mol Biol 90:447–452.

Weise M.J. and D.P. Costa. 2007. Total body oxygen stores and physiological diving capacity of California sea lions as a function of sex and age. J Exp Biol 210:278–228.

Wu C., J. Ye, J. Gao, L. Chen, and Z. Lu. 2016. The effects of dietary carbohydrate on the growth, antioxidant capacities, innate immune responses and pathogen resistance of juvenile Black carp *Mylopharyngodon piceus.* Fish Shellfish Immunol 49:132–142.

Zhang Z., R. Hou, J. Lan, H. Wang, H. Kurokawa, Z. Takatsu, T. Kobayashi, et al. 2016. Analysis of the breast milk of giant pandas (*Ailuropoda melanoleuca*) and the preparation of substitutes. J Vet Med Sci 78:747–754.

Zipurksy A., E. Brown, J. Palko, and E.J. Brown. 1983. The erythrocyte differential in newborn infants. Am J Pedriatr Hematol Oncol 5:45–51.

ARTICULO II

En esta sección se presenta el segundo artículo derivado de la tesis, con título “Climatic anomaly affects the immune competence of California sea lions”, que fue publicado en el 2017 en PLoS ONE

PLOS | ONE

RESEARCH ARTICLE

Climatic anomaly affects the immune competence of California sea lions

Marina Banuet-Martínez[1], **Wendy Espinosa-de Aquino**[1], **Fernando R. Elorriaga-Verplancken**[2], **Adriana Flores-Morán**[1], **Olga P. García**[3], **Mariela Camacho**[3], **Karina Acevedo-Whitehouse**[1,4]*

1 Unit for Basic and Applied Microbiology, School of Natural Sciences, Autonomous University of Queretaro, Queretaro, Mexico, **2** Department of Fisheries and Marine Biology, Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional (CICIMAR-IPN), La Paz, Mexico, **3** Department of Human Nutrition, School of Natural Sciences, Autonomous University of Queretaro, Queretaro, Mexico, **4** The Marine Mammal Center, Sausalito, California, United States of America

* karina.acevedo.whitehouse@uaq.mx

Abstract

The past decades have been characterized by a growing number of climatic anomalies. As these anomalies tend to occur suddenly and unexpectedly, it is often difficult to procure empirical evidence of their effects on natural populations. We analysed how the recent sea surface temperature (SST) anomaly in the northeastern Pacific Ocean affects body condition, nutritional status, and immune competence of California sea lion pups. We found that pup body condition and blood glucose levels of the pups were lower during high SST events, although other biomarkers of malnutrition remained unchanged, suggesting that pups were experiencing early stages of starvation. Glucose-dependent immune responses were affected by the SST anomaly, specifically, pups born during high SST events had lower serum concentrations of IgG and IgA, and were unable to respond to an immune challenge. This means that not only were pups that were born during the SST anomaly less able to synthesize protective antibodies; they were also limited in their ability to respond rapidly to non-specific immune challenges. Our study provides empirical evidence that atypical climatic conditions can limit energetic reserves and compromise physiological responses that are essential for the survival of a marine top predator.

Introduction

During the boreal winter of 2013 a sudden increase in sea surface temperature (SST) was detected in the Gulf of Alaska [1,2]. This anomaly, referred to as 'The Blob', extended gradually southwards. The SST anomaly was further impacted by an El Niño event, confirmed in June 2015 [3]. These events led to an unprecedented 2 to 5°C increase above the historical average in the SST [2]. By summer 2015 the anomalously high SST had encompassed the entire northeastern Pacific, including the southernmost tip of the Baja California Peninsula, Mexico. Specifically, the SST anomaly was on average 1°C higher in 2015 than in 2014 for the central part of Baja California, where the San Benito Archipelago is located, and in some months exceeded

Citation: Banuet-Martínez M, Espinosa-de Aquino W, Elorriaga-Verplancken FR, Flores-Morán A, García OP, Camacho M, et al. (2017) Climatic anomaly affects the immune competence of California sea lions. PLoS ONE 12(6): e0179359. https://doi.org/10.1371/journal.pone.0179359

Editor: Ashlesh K Murthy, Midwestern University, UNITED STATES

Received: February 22, 2017

Accepted: May 30, 2017

Published: June 28, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by a grant from the Autonomous University of Queretaro (Fund for the Advancement of Scientific Research, 142908; http://www.uaq.mx/dip/index.php/fofi-uaq) and partly funded by the Mexican Consejo Nacional de Ciencia y Tecnología (CONACYT; http://www.conacyt.gob.mx), grant numbers: CB-181876 and CB-153530. Data from the 2012 cohort was collected during a study funded by CONACYT grant number CB-153530. AFM, WEA and MBM are funded by CONACYT graduate scholarships 287579, 369084, and 778607, respectively. Publication fees were covered by support from the Programa de Fortalecimiento a la Calidad Educativa, PFCE 2016 (Mexico). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

4°C [4]. The duration of the anomalies was also extended in 2015, with six of the months reaching over two standard deviations of the SST recorded between 1988 and 2014 [5].

High SSTs alter the primary productivity of the marine environment, which in turn affects all trophic levels [6,7], with disastrous consequences for sea birds and marine mammals [8,9]. One of the most dramatic effects of high SST was observed during the 1982–1983 El Niño, which led to a marked reduction of the Galapagos fur seal (***Arctocephalus galapagoensis***) population [10]. The high SST driven by 'The Blob' has already led to ecosystem alterations and biomass changes, reductions in the abundance of fishes [11], and increased stranding events of young California sea lions (***Zalophus californianus***, hereafter CSL) [12,13]. 'The Blob' was even related to the occurrence of a large harmful algal bloom that affected CSL as well as other marine mammals [14].

The CSL is an otariid pinniped that is distributed throughout the northeast Pacific. Its coastal feeding habits [15,16] make it particularly vulnerable to declines in prey availability [8]. As adult female CSL tend to procure prey less than 100 km from their breeding sites [17], well within the area affected by 'The Blob', diminished prey availability forces them to extend their foraging trips in order to feed [17,18], and results in consumption of prey of lower nutritional and energetic value, such as shortbelly rockfish, instead of the typically preferred sardines or anchovies in areas adjacent to California [12]. Such behavioural modifications will likely have consequences for pregnant or lactating CSLs (which are already undergoing an energetically-demanding process) [19], as well as for their offspring.

If suboptimal maternal nutrition means that fewer resources are available for developing pups, then costly physiological processes, such as immune responses, could be hampered. Maintaining and deploying the immune effectors needed for survival is greatly demanding of an individual's resources [20]. For instance, in the house sparrow, ***Passer domesticus***, cell-based immune responses to a mitogen requires the investment of energy equivalent to that needed to produce half an egg [21], and during bouts of fever, each 1°C rise in the core temperature leads to a 10–15% increase in metabolic rate [22]. Taking into account the atypical oceanographic conditions brought on by 'The Blob' and El Niño, and its impact on adult female CSL foraging behaviour [18,23], and diet [12], it is reasonable to assume that newborn pups did not experience 'normal' nursing during the SST anomaly, in terms of the amount and quality of milk received. There is some indication that this is indeed the case, as CSL pups born in 2015 in the Mexican north Pacific had lower mass than those born in 2014, when the SST anomaly was less pronounced in that region [5,18]. While there are published studies on the effects of maternal condition, foraging requirements and fasting times on milk quality [24–26], we are unaware of any study on variations in quality of otariid maternal milk caused by atypical oceanographic conditions. However, seasonal hunger has been shown to lead to a temporary decrease in production [27] and quality [28] of human milk, and maternal nutritional status can hinder neonatal development, growth, and survival [29]. If this were the same for the CSL, the abnormally high SST could impact pup fitness severely, particularly as offspring rely entirely on maternal milk during their first six months of age, and continue to nurse for most of their first year [30].

Here, we studied whether CSL pups born during the anomalous SST events had fewer resources available to elicit adequate immune responses than pups born during a year with normal SST conditions. To address this research question, we estimated pup condition, quantified blood biomarkers of malnutrition, and examined different measures of immune function in pups born during the 2014 and 2015 breeding seasons (abnormal SST events), and we compared them to those of pups born during a year of normal SST conditions. Based on resource allocation theory [31,32], we predicted that pups with a higher body condition and better nutritional status would have *i*) more marked ***in vivo*** responses to a mitogenic challenge, *ii*) higher immunoglobulin concentrations, and *iii*) fewer blood values outside of the normal clinical range.

Materials and methods

Study area and species

As part of on-going studies on pinniped ecology, during the breeding season of 2014 (mid July) we sampled 28 CSL pups at the San Benito Archipelago (SBA; 28˚18'N, 115˚34'W) in the Mexican Northern Pacific. SBA is comprised by a group of three islands (named East, Middle, and West) located 270 km southeast of Guadalupe Island and 75 km northwest of the Baja California Peninsula, Mexico. All sampling was carried out in West Benito Island. In 2015, aware that the high SST associated with 'The Blob' and El Niño events had reached SBA, we returned to the field site and sampled 33 CSL pups born during that breeding season. However, even though the SST anomaly was less pronounced in 2014 than in 2015 as reported for the study region by the NCEP/NCAR Reanalysis Project [33] of the Earth System Research Laboratory, National Oceanic and Atmospheric Administration (NOAA), it certainly could not be considered a typical year (Fig 1). Thus, we considered 2014 as a 'less-severe' SST anomaly for the region. Since we did not have immune, haematological, and serological data from pups born at SBA during normal SST conditions, we used data from 23 clinically healthy CSL pups of the same age (6–8 weeks of age) born in 2012 at the rookery found in Granito Island (29˚33' N, 113˚32' W) as reference values. These pups were born during a year that was characterized by normal SST [5], and their capture, handling, and sampling as well as all haematological and serological analyses were conducted exactly as described above.

Capture, handling and sampling

Sea lion pups were captured with hoop nets and were restrained manually during sampling. For each pup, mass and total length were recorded, and blood samples were collected. The mass was determined using a vertical hanging scale (0.1 kg precision) and the length from the tip of the tail to the nose was measured with a tape measure (1 mm precision).

Visual inspection by a veterinarian ascertained the general health status of each pup. Two blood samples (7 ml each) were collected from the caudal gluteal vein using an 18-gauge needle and vacuum tubes (Vacutainer, BD Biosciences, USA), one with EDTA and one without any preservative. Blood was kept in an upright position in a cooler, protected from direct sunlight, and was centrifuged at 3200 rpm for 10 min within three hours since the time of collection. Serum was separated and cryopreserved in a liquid nitrogen container until further analysis.

Sampling was conducted by approval of the Bioethics Committee and IACUC of the Autonomous University of Queretaro. All procedures were carried out under permits SGPA/DGVS/11744/13 and SGPA/ DGVS/00195/15 issued by the Dirección General de Vida Silvestre (DGVS) of the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT).

Body condition and nutritional status

We calculated mass per unit length and obtained the scaled mass index for each pup [34]. This proxy of body condition was used because the relationship between energetic reserves and body size is complex, and this measure takes into account the scaling between body components and body size [34].

We quantified the concentration of different blood biomarkers of malnutrition [35]. Namely, we measured glucose, triglycerides, total cholesterol, HDL, total proteins, albumin, creatinine, and blood urea nitrogen. Parameters were measured by spectrophotometry using

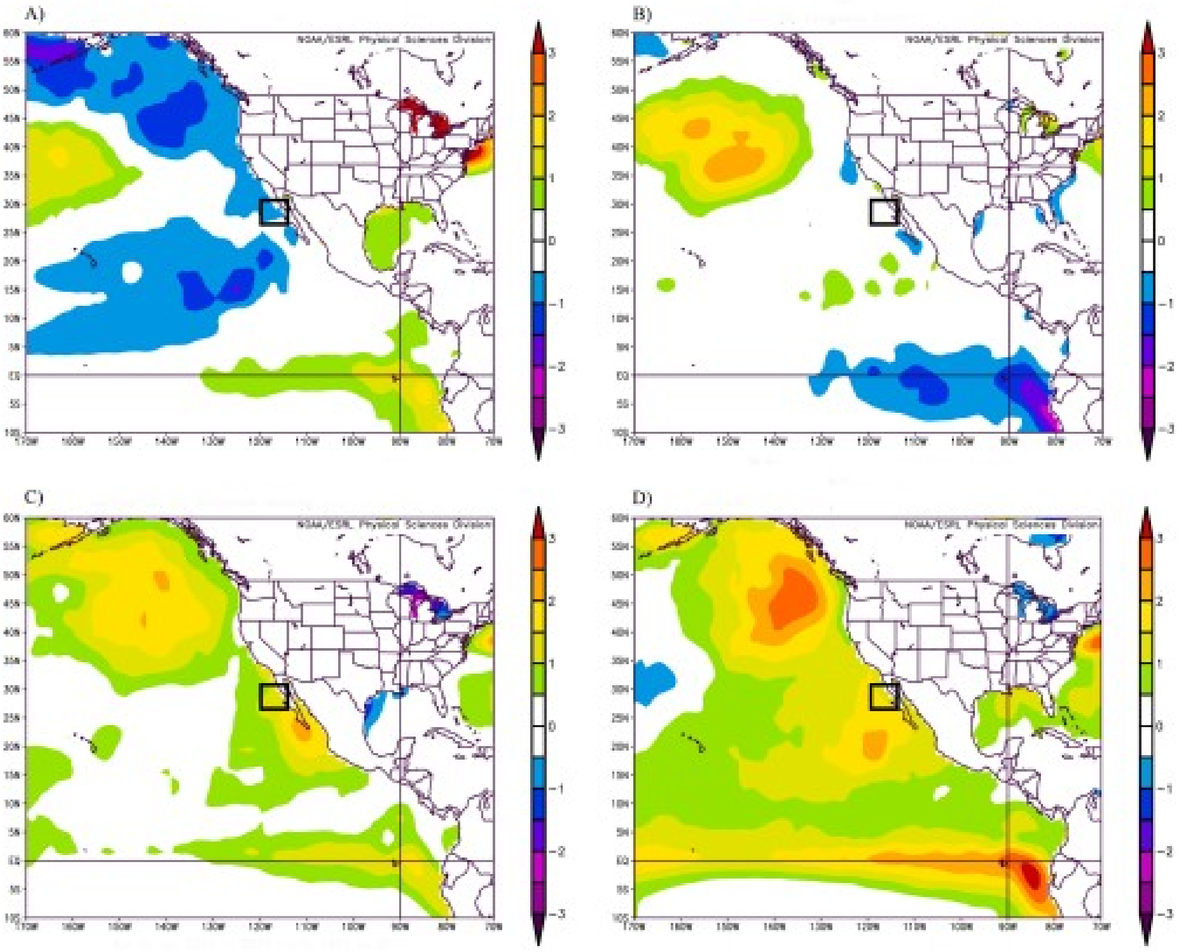

Fig 1. **Sea surface temperature anomaly (˚C) in the northeastern Pacific Ocean.** Panels show the average of values recorded April 1st to July 31st for (**A**) 2012, (**B**) 2013, (**C**) 2014, and (**D**) 2015. The location of the San Benito Archipelago is indicated within the square. Images provided by the NOAA/ESRL Physical Sciences Division, Boulder Colorado from their Web site at http://www.esrl.noaa.gov/psd/. The plots were created by using data collected by the NCEP/NCAR Reanalysis Project [33].

https://doi.org/10.1371/journal.pone.0179359.g001

an automated biochemistry analyser (Spin Lab 120, Spinreact) and commercially available kits (Spinreact S.A.U. Ctra Santa Coloma 7, E-17176 Sant Esteve de Bas, Spain). The biochemistry analyser was designed for human use and was calibrated prior to running the samples using a calibrator from the manufacturer. Two controls of lyophilized human serum were run together with the CSL samples for each assay. If a control failed, the assay was repeated.

Assessment of immune parameters

White blood cell counts. Total white blood cell (WBC) counts were performed less than 8 h after blood collection using a haemocytometer (Optic Labor, CA, USA). We prepared three blood smears per sample and fixed them in 90% methanol before staining with Wright solution. For each smear, we determined the different WBC populations by counting the number of neutrophils, band neutrophils, hypersegmented neutrophils, lymphocytes, monocytes, eosinophils, and basophils in 100 cells. Absolute numbers for each leucocyte type were calculated by multiplying the total white blood cell count by the percentage of the each leucocyte type.

Based on the WBC reference values (mean ± 2 SD; S1 Table) previously calculated for clinically healthy pups born during normal SST conditions, we determined the presence (or absence) of clinical indications of disease, namely leucocytosis, neutrophilia, left-shift, monocytosis, lymphocytosis, lymphopenia, eosinophilia and basophilia [36].

Immunoglobulin concentrations. Immunoglobulin (Ig) isotypes A and M were quantified by indirect ELISA using mouse anti-dog IgA horseradish peroxidase (HPR) conjugate and mouse anti-dog IgM HPR as primary antibodies, and goat anti-mouse IgG-HPR, Novex) as a secondary antibody. The Mucosal Immunology Laboratory of the Veterinary School at Bristol University, UK provided the primary antibodies. For details on the protocols used see Supporting Information. IgG concentrations were measured with a protein A ELISA as reported previously [37], with slight modifications (see Supporting Information). Absorbance was measured in an ELISA microplate reader (BioRad, USA) at 450 nm. For each isotype, absorbance readings were interpolated on a standard curve using dog serum (Bethyl Laboratories, USA) as a reference. All reactions were run in triplicate.

PHA challenge. We examined the pups' ability to react to intradermal injections of phytohemagglutinin (PHA), a plant-derived lectin that stimulates T lymphocyte mitogenesis [38] and causes local inflammation as a direct result of tissue damage [39]. The challenges were conducted as done previously [40]. Briefly, for each pup we measured the thickness of the webbing between the second and third digits of both hind flippers and inoculated 100 μl of 1 mg/ml of PHA intradermally into the right flipper, and a control (100 μl of sterile saline solution) into the left flipper. Webbing thickness at the inoculation site was measured after 4 h [40]. All measurements were taken in triplicate to the nearest 0.01 mm with a thickness gauge (Mitutoyo, USA). The response to PHA was calculated as the difference between the change in median thickness of the left flipper and the change in median thickness of the right flipper. This challenge was conducted only on pups born in 2015, and data was compared to that recorded for pups of the same age, born in 2012, which had been challenged using the same protocol.

Statistical analyses

We initially explored our dataset graphically to establish the spread and distribution of the data. Continuous response variables that deviated from the normal distribution were examined with Cullen and Frey graphs to determine their distribution. We built a series of independent generalized linear models (GLM) to examine i) variations in the measured parameters amongst birth cohorts (2014: less-severe SST anomaly; 2015 severe SST anomaly; 2012 normal SST conditions), and ii) explore the relationship between immune-related variables and condition or biomarkers of nutritional/metabolic status. Response variables that had a beta distribution (IgG, IgA, IgM, triglycerides, and all white blood cell populations) were modelled with a quasibinomial error distribution and logit link. Dichotomous response variables (presence of leucocytosis, neutrophilia, left-shift, monocytosis, lymphocytosis, lymphopenia, eosinophilia and basophilia) were modelled with a binomial error distribution and logit link. All other variables were normally distributed and were modelled with a Gaussian error distribution. Tukey HSD tests were used to further examine differences in variables between years. Due to unequal sample size and asymmetric variance, differences in webbing thickness (measure of the response to the PHA challenge) of pups born in 2015 and 2012 were examined with a

Kruskal-Wallis rank sum test. Contingency tables were built in order to investigate whether the prevalence of each clinical indicator of deviation from health varied amongst birth cohorts. We used Fisher exact tests to determine the level of significance. All analyses were performed in R version 3.3.1 [41].

Results

Variation in body condition and nutritional parameters

Blood levels of cholesterol, triglycerides, HDL, total protein, albumin, creatinine, and urea were within the reference values (S2 Table), and did not vary amongst birth cohorts. In contrast, mean glucose levels were 15% lower in pups born under atypical SST conditions ($F_{2,70}$ = 4.35, p = 0.013; Fig 2a). The severity of the SST event did not affect the blood concentration of glucose (2014 = 2015; Tukey HSD, p = 0.14).

Body condition was significantly lower for pups born during the years of high SST conditions than during 2012 ($F_{2,79}$ = 20.37, p = $7.35\text{x}10^{-08}$; Fig 2b), and those born in 2015 had a lower body condition than those born in 2014 (Tukey HSD, p = 0.02). Glycaemia was predicted by pup body condition, regardless of differences between years ($F_{2,71}$ = 7.68, p = 0.007; Fig 2c). None of the other biomarkers of nutritional and metabolic status were related to body condition.

Variation in immune parameters

Total WBC counts of the pups born at SBA were similar amongst birth cohorts, regardless of the severity of the SST anomaly, and did not differ from the values recorded for healthy pups of the same age that were born under normal environmental conditions (GLM; $F_{2,79}$ = 0.26, p = 0.772). Most of the differential WBC counts of pups born in 2014 and 2015 were within the normal ranges recorded for healthy pups and did not vary markedly from those counts recorded for pups born in 2012 (S1 Table). However, basophils were virtually absent in pups born in 2015, compared to pups born in 2014 and 2012 (GLM; $\chi^2_{2,79}$ = 4.84, p = 0.01; Fig 3a), and pups born in 2014 and 2015 had almost nine times more band neutrophils (mean = 5618.1) than pups born in 2012 (mean = 662.99) (GLM; $\chi^2_{2,79}$ = 15.05, p = $2.2\text{x}10^{-16}$; Fig 3b). Furthermore, 50% of the pups born in 2015 had hypersegmented neutrophils, in contrast to

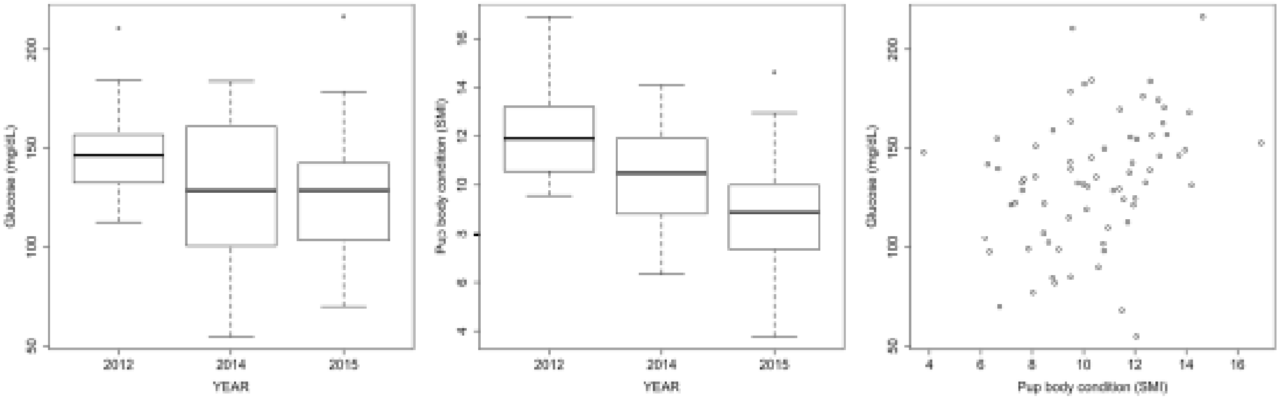

Fig 2. Indicators of nutritional status of California sea lion, *Zalophus californianus*, pups born during normal (2012) and atypical sea surface temperature conditions. A) Blood glucose concentration, B) Pup body condition calculated as the Scaled Mass Index, C) Blood glucose levels were related to pup body condition.

https://doi.org/10.1371/journal.pone.0179359.g002

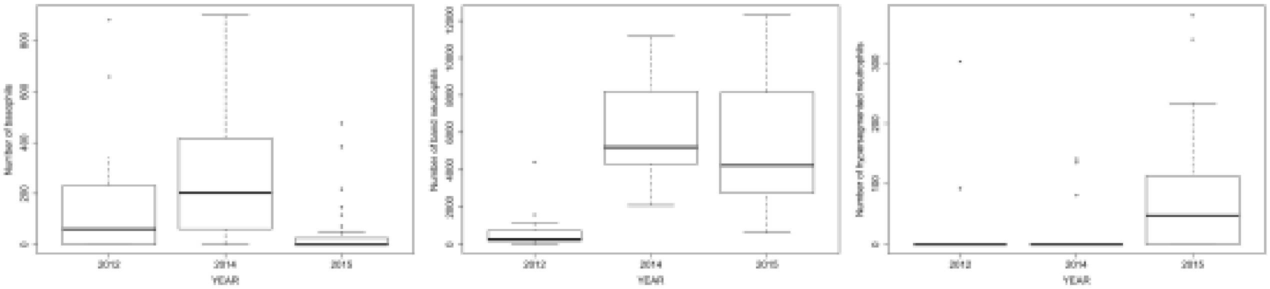

Fig 3. White blood cell counts of California sea lion, *Zalophus californianus*, pups that differed between those born during normal (2012) and atypical (2014 and 2015) sea surface temperature conditions. A) Basophils, B) Band neutrophils, C) Hypersegmented neutrophils.

https://doi.org/10.1371/journal.pone.0179359.g003

what was observed for the other birth cohorts (2014: 0.8%; 2012: 0.92%), and these cells were significantly more abundant in pups born in 2015 (GLM; $F_{2,80} = 28.52$, $p = 0.004$; Fig 3c). None of the pups born in 2014 and 2015 had evidence of lymphopenia. The proportion of CSL pups with a left-shift was significantly higher for pups born during atypical years (2014: 100%, 2015: 90.6%, 2012: 4.3%; Pearson's Chi-sq = 68.05; $p = 0$), and a similar pattern was seen for eosinophilia (2014: 29.6%, 2015: 21.9%, 2012: 4.3%; Pearson's Chi-sq = 4.68; $p = 0.02$). The prevalence of leucocytosis, lymphocytosis, lymphopenia, monocytosis, neutrophilia, and basophilia did not vary amongst years.

IgM levels did not vary amongst birth cohorts, but the concentrations of IgG and IgA were significantly lower in pups born during an SST anomaly than in a normal year (GLM; IgG: $\chi^2_{2,70} = 6.89$, $p = 0.002$; IgA: $\chi^2_{2,70} = 30.06$, $p = 3.77\times10^{-10}$; Fig 4).

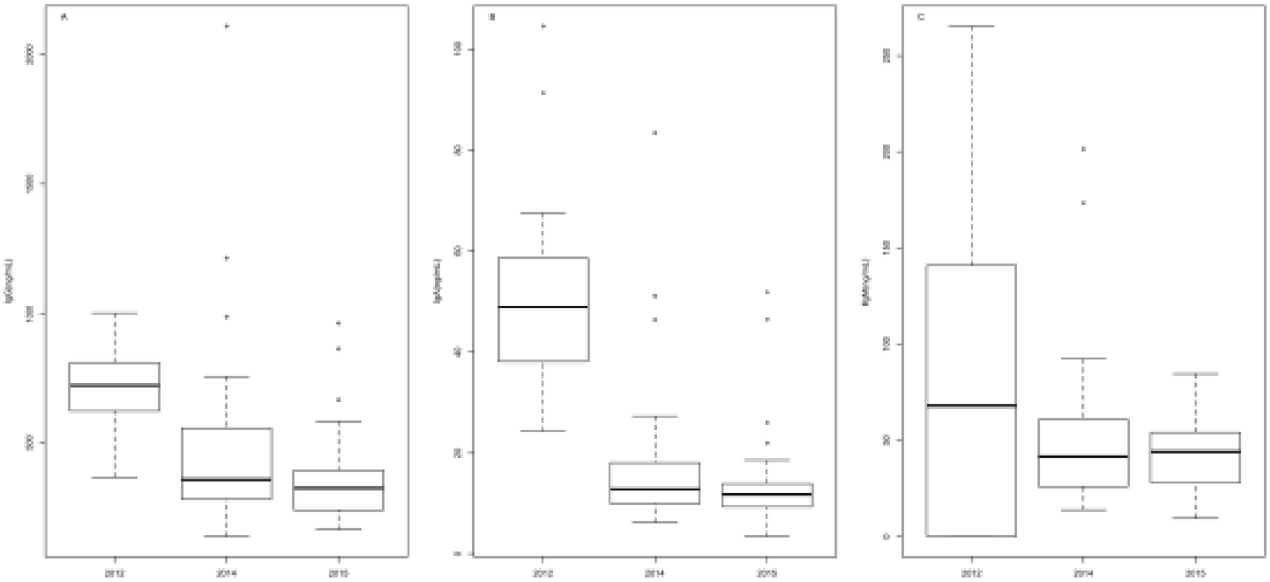

Fig 4. Serum antibody levels of California sea lion, *Zalophus californianus*, pups born under normal (2012) and atypical (2014 and 2015) sea surface temperature conditions. A) IgG, B) IgA, C) IgM.

https://doi.org/10.1371/journal.pone.0179359.g004

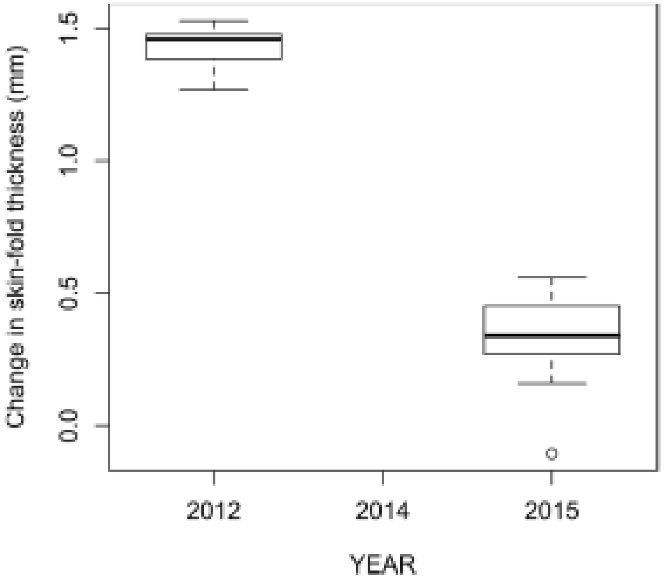

Fig 5. Skin-fold thickness in response to PHA challenge. Response to PHA in California sea lion, *Zalophus californianus*, pups born during normal (2012) and atypical (2015) sea surface temperature conditions.

https://doi.org/10.1371/journal.pone.0179359.g005

The response to the PHA was 4.13 times lower for pups born in 2015 than in 2012 (Kruskal-Wallis; $\chi^2 = 16.57$, df = 1, $p = 4.7\text{x}10^{-5}$; Fig 5).

Body condition, nutritional status and immune effectors

Pups with a better body condition tended to have a higher serum concentration of IgA (GLM; $F_{1,70} = 7.42$, $p = 0.008$) and both IgG and IgA concentrations increased with blood glucose level (GLM; IgG: $F_{2,71} = 12.86$, $p = 0.0006$; IgA: $F_{2,71} = 14.57$, $p = 0.0003$). The response to PHA also increased in relation to blood glucose (GLM; $F_{2,72} = 13.96$, $p = 7.55\text{x}10^{-06}$). None of the other biomarkers of nutrition were significantly associated with any of the immune effectors.

Discussion

The immune system is indispensable for the survival of any individual. In order to function adequately, the effectors that participate in mounting an immune response require nutrients and energy [20,32]. Here, we tested the hypothesis that if the abnormally high SST conditions caused by 'The Blob' [1,2], and a simultaneous El Niño event [3,5] posed a nutritional and energetic challenge for pregnant and lactating CSL females [17,18], then their offspring were likely to have lower body condition, altered nutritional status and impaired immune responses. As we did not have data for pups born at SBA during normal SST conditions, we used data collected from pups of the same age that were born during a normal year at a rookery located in the midriff region of the Gulf of California. While not ideal, this data was considered valid to use as reference data, as published data on haematology, blood chemistry, and serology of CSL focuses mainly on older animal, and mostly on stranded or captive individuals [42].

We found that body condition was significantly lower in pups born during the atypical climatic conditions, and was even lower for pups born in 2015, when the SST was higher than in

2014 [3,5]. However, except for glucose, which was 15% lower in pups born under atypical SST conditions, the biomarkers selected to assess nutritional/metabolic status were within the reference values used in this study and also fell within the range reported for young CSL [36]. This implies that rather than malnutrition, these pups may have experienced undernutrition, an early stage of starvation in which an individual has not yet switched to gluconeogenesis, that if not corrected will lead to malnutrition [43]. Finding that glycaemia was directly predicted by pup body condition further strengthens this possibility.

None of the pups showed any clinical signs of disease at physical examination, and most differential WBC counts were within the reference values here used (pups born in Granito in 2012), as well as within the normal range reported for yearling CSL [36], suggesting that at the time of sampling, the health of the pups was comparable amongst birth cohorts. One exception was eosinophils, which tended to be more abundant in pups born in 2014 and 2015 than those born in 2012. Eosinophilia has been related to domoic acid intoxication [44], and it could be argued that the CSL pups born during the high SST conditions were affected by mammary transmission of domoic acid from lactating mothers that fed in areas where toxic blooms of domoic acid-producing algae, ***Pseudo-nitzschia*** spp., were occurring [45]. The high SST conditions of 2014 and 2015 are known to have favoured blooms of ***Pseudo-nitzschia australis*** along the western coast of the US, causing considerable damage to CSL and other marine mammals [14]. However, to the best of our knowledge, there has been no evidence of domoic acid intoxication in sea lions at SBA, nor have blooms of ***Pseudo-nitzschia*** been reported to occur within the feeding range of SBA adult females during the 2014 and 2015 anomalies. Also, during the duration of our field trip we failed to observe abortions, as would be expected during ***in utero*** toxicity if pregnant females fed in locations where ***Pseudo-nitzschia*** blooms were occurring [46]. Except for one adult male that was displaying an unusual "rolling" movement along the beach, we did not observe seizures or altered behaviours associated with DA intoxication [47] in any adult female, juvenile or pup during the study years. In that sense, while possible, it is unlikely that the CSL pups were affected by DA intoxication.

In addition to eosinophilia, the second exception to the largely normal leukogram was the number of basophils, which were virtually absent in pups born in 2015, compared to pups born in 2014 and 2012. While circulating basophil numbers are typically low compared to other WBC types, an absence of basophils can be indicative of adrenocortical hyperfunction due to stress [48]. It is possible that pups born in 2015 might have experienced high levels of stress due to suboptimal nursing, or could have been affected ***in utero*** by maternal levels of stress [49], partially explaining the unusually high number of abortions recorded at SBA at the beginning of May 2015, prior to the breeding season (unpublished data). Furthermore, pups born at SBA in 2014 and 2015 had almost nine times more band neutrophils than pups born in 2012. The presence of these immature cells tends to indicate that the demand for neutrophils exceeds its supply. As we found no clinical evidence of malaise, and the other WBC populations in these pups were within the ranges, it is possible that their presence was an early indication of subclinical infection [50]. Alternatively, the high numbers of band neutrophils could be undernutrition, as has been reported for starving juvenile Northern elephant seals, ***Mirounga angustirostris*** [51]. The fact that half of the pups born in 2015, during the most severe high SST event, had hypersegmented neutrophils which are common during nutrient-deficiency anaemia in human neonates [52], would appear to support this possibility.

We hypothesized that if pups were experiencing suboptimal nutrition, immunoglobulin synthesis might be constrained. We found evidence to support this, as the concentration of IgG and IgA (but not IgM) was markedly lower in pups born during 'atypical' years. Given that pup blood glucose levels were found to be altered by the high SST events, the observed pattern was exactly as would be predicted because class-switching of IgG and IgA occurs via T-cell dependent (glucose-dependent) and T-cell independent pathways [53], while IgM class switching is T-cell independent [54].

If nutritional and energetic impoverishment impairs pups' body condition and limits glucose-dependent immunoglobulin class switching, other T-cell dependent immune responses could also be affected. Indeed, we found that responses to a mitogenic challenge were severely impaired in individuals born under atypical environmental conditions. The apparent inability of these pups to respond to PHA is most likely due to insufficient energetic reserves, as was evidenced by the direct association between the magnitude of the swelling response to PHA and blood glucose concentration. This means that not only were the pups that were born during the SST anomaly less able to synthesize protective antibodies; they were also limited in their ability to respond rapidly to nonspecific immune challenges. Taken together, these results constitute evidence that atypical climatic conditions can encumber the energetic reserves and immune competence of neonates, thus decreasing their fitness.

Conclusions

The consequences of having suboptimal immune responses could be devastating to the entire CSL 2014 and 2015 cohorts, particularly if a highly virulent pathogen were to 'enter' the population. Even in absence of such a challenge, without proper nutrition, pups are likely to be unable to elicit responses against opportunistic pathogens, and mortality would expectedly be high. We returned to SBA in February 2016 and found that the entire CSL colony was reduced by 77%, relative to the previous year during the same dates (total count: 584 in 2016 *vs*. 2,555 in 2015). Extremely few live pups were found at the archipelago (343 in 2016 *vs*. 1,567 in 2015) and most of those that were present were severely emaciated and weak. An unusual high number of carcasses were also recorded. It is too early to understand the impacts that the abnormal oceanographic conditions will have on CSL pups later in their life, but based on life-history theory, their fitness would expectedly be poor. Furthermore, as the high SST conditions encompassed the northeastern Pacific, the entire CSL population could be impacted, as has been reported for other pinnipeds during past El Niño events [10].

In conclusion, we have shown that abrupt and unexpected environmental changes can affect key physiological components. The observed effects of the abnormally high SST linked to The Blob and El Niño are unlikely to be limited to the CSL; the entire northeastern Pacific ecosystem could potentially be vulnerable. Under the current climatic scenario, systematic surveys within this region would be invaluable to determine the full scope of effects and assess their wider and long-term consequences.

Supporting information

S1 Table. Reference values of total and differential white blood cell (WBC) counts from clinically healthy California sea lion, *Zalophus californianus*, pups born in 2012 at Granito Island in the Gulf of California. The table also shows the mean and standard deviation of each cell type for pups born in 2014 and 2015 at the San Benito Archipelago.
(PDF)

S2 Table. Reference values of blood chemistry parameters from clinically healthy California sea lion, *Zalophus californianus*, pups born in 2012 at Granito Island in the Gulf of California. The table also shows the mean and standard deviation of each cell type for pups born in 2014 and 2015 at San Benito Archipelago.
(PDF)

S1 File. Data used for the analyses presented in this paper.
(PDF)

S2 File. More detailed description of methods used.
(PDF)

Acknowledgments

We thank the Abalone Fishermen Cooperative for their help during fieldwork. Luis Soto-Garcia and Cecilia Barragán-Vargas assisted during pup captures and sample processing on site. We are indebted to Erika Vargas Perusquia for her support with grant administration. FREV thanks the Instituto Politécnico Nacional for support received through the Contracting Excellence Program and fellowship EDI.

Author Contributions

Conceptualization: KAW.

Data curation: MBM WEA.

Formal analysis: MBM WEA AFM OG MC.

Funding acquisition: KAW FREV.

Investigation: MBM WEA.

Methodology: KAW.

Project administration: KAW.

Resources: KAW OG FREV.

Supervision: KAW.

Validation: KAW.

Visualization: MBM KAW.

Writing – original draft: MBM KAW.

Writing – review & editing: KAW FREV OG.

References

1. Bond NA, Cronin MF, Freeland H, Mantua N. Causes and impacts of the 2014 warm anomaly in the NE Pacific. Geophys Res Lett. 2015; 42: 3414–3420. https://doi.org/10.1002/2015GL063306
2. Kintisch K. "The Blob" invades Pacific, flummoxing climate experts. Science 2015; 348: 17–18.
3. National Oceanic and Atmospheric Administration. El Niño/Southern Oscillation (ENSO) Diagnostic Discussion. http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/enso_advisory/ensodisc.html.
4. Leising AW, Schroeder ID, Bograd SJ, Abell J, Durazo R, Gaxiola-Castro RG, et al. State of the California Current 2014–15: Impacts of the warm water "Blob". California Coop Fisheries Invest Rep. 2015; 56:31–68.
5. Robinson CJ. Evolution of the 2014–2015 sea surface temperature warming in the central west coast of Baja California, Mexico, recorded by remote sensing. Geophys Res Lett. 2016; 43:7066–7071. https://doi.org/10.1002/2016GL069356
6. Easterling DR, Meehl GA, Parmesan C, Changnon SA, Karl TR, Mearns LO. Climate extremes: observations, modeling, and impacts. Science 2000; 289: 2068–2074. https://doi.org/10.1126/science.289.5487.2068 PMID: 11000103

7. Alestra T, Schiel DR. Impacts of local and global stressors in intertidal habitats: Influence of altered nutrient, sediment and temperature levels on the early life history of three habitat-forming macroalgae. J Exp Mar Biol Ecol. 2015; 468: 29–36 http://dx.doi.org/10.1016/j.jembe.2015.03.017
8. Barber RT, Chavez FP. Biological consequences of El Niño. Science 1983; 222: 1203–1210. https://doi.org/10.1126/science.222.4629.1203 PMID: 17806711
9. Devney CA, Short M, Congdon BC. Sensitivity of tropical seabirds to El Niño precursors. Ecology 2009; 90: 1175–1183. https://doi.org/10.1890/08-0634.1 PMID: 19537539
10. Trillmich F, Limberger D. Drastic effects of El Niño on Galapagos pinnipeds. Oecologia 1985; 67: 19–22. https://doi.org/10.1007/BF00378445 PMID: 28309839
11. Chust G, Allen JI, Bopp L, Schrum C, Holt J, Tsiaras K, et al. Biomass changes and trophic amplification of plankton in a warmer ocean. Global Change Biol. 2014; 20: 2124–2139. https://doi.org/10.1111/gcb.12562 PMID: 24604761
12. McClatchie S, Field J, Thompson AR, Gerrodette T, Lowry M, Fiedler PC, et al. Food limitation of sea lion pups and the decline of forage off central and southern California. R Soc Open Sci. 2016; 3(3): 150628 https://doi.org/10.1098/rsos.150628 PMID: 27069651
13. National Oceanic and Atmospheric Administration. FAQs on the 2013 California Sea Lion UME Investigation. 2014. http://www.nmfs.noaa.gov/pr/health/mmume/casealion2013_investigation.htm.
14. McCabe RM, Hickey BM, Kudela RM, Lefebvre KA, Adams NG, Bill BD et al. An unprecedented coastwide toxic algal bloom linked to anomalous ocean condition. Geophys Res Lett. 2016; 43: 366–376. https://doi.org/10.1002/2016GL070023 PMID: 27917011
15. Lowry MS, Stewart BS, Heath CB, Yochem PK, Francis JM. Seasonal and Annual Variability in the Diet of California Sea Lions *Zalophus californianus* at San Nicolas Island, California, 1981–86. Fish Bull. 1990; 89: 331–336.
16. Garcia-Rodriguez FJ, Aurioles-Gamboa D. Spatial and temporal variation in the diet of the California sea lion (*Zalophus californianus*) in the Gulf of California, Mexico. Fish Bull. 2004; 102: 47–62.
17. Kuhn CE, Costa DP. Interannual variation in the at-sea behavior of California sea lions (*Zalophus californianus*). Mar Mamm Sci. 2014; 30: 1297–1319. https://doi.org/10.1111/mms.12110
18. Elorriaga-Verplancken FR, Sierra-Rodriguez GE, Rosales-Nanduca H, Acevedo-Whitehouse K, Sandoval-Sierra J. Impact of the 2015 El Niño-Southern Oscillation on the Abundance and Foraging Habits of Guadalupe Fur Seals and California Sea Lions from the San Benito Archipelago, Mexico. PLoS One. 2016; 11(5): e0155034. https://doi.org/10.1371/journal.pone.0155034 PMID: 27171473
19. Speakman JR. The physiological costs of reproduction in small mammals. Philos Trans R Soc Lond B Biol Sci. 2008; 363: 375–398. https://doi.org/10.1098/rstb.2007.2145 PMID: 17686735
20. McDade TW. Life history, maintenance, and the early origins of immune function. Am J Hum Biol. 2005; 17: 81–94. https://doi.org/10.1002/ajhb.20095 PMID: 15612049
21. Martin LB, Scheuerlein A, Wikelski M. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc R Soc B Biol Sci. 2003; 270: 153–158. https://doi.org/10.1098/rspb.2002.2185 PMID: 12590753
22. Roe CF, Kinney JM. The Caloric Equivalent of Fever II. Influence of Major Trauma. Ann Surg. 1965; 161: 140–147. PMID: 14252624
23. Weise MJ, Costa DP, Kudela RM. Movement and diving behavior of male California sea lion (*Zalophus californianus*) during anomalous oceanographic conditions of 2005 compared to those of 2004. Geophys Res Lett. 2006; 33: L22S10. https://doi.org/10.1029/2006GL027113
24. Leung ES, Augé AA, Chilvers BL, Moore AB, Robertson BC. Foraging behaviour of juvenile female New Zealand sea lions (*Phocarctos hookeri*) in contrasting environments. PLoS One. 2013; 8(5): e62728. https://doi.org/10.1371/journal.pone.0062728 PMID: 23671630
25. Georges J-Y, Groscolas R, Guinet C, Robin JP. Milking strategy in subantarctic fur seals *Arctocephalus tropicalis* breeding on Amsterdam Island: evidence from changes in milk composition. Physiol Biochem Zool. 2001; 74:548–559. https://doi.org/10.1086/322164 PMID: 11436139
26. Rietz-Sapriza FG, Duignan PJ, Chilvers BL, Wilkinson IS, Lopez-Villalobos N, Mackenzie DD, MacGibbon A, Costa DP, Gales N. Interannual and individual variation in milk composition of New Zealand sea lions (*Phocarctos hookeri*). J Mammal. 2012; 93:1006–1016. https://doi.org/10.1644/11-MAMM-A-220.2
27. Whitehead RG. Nutrition and lactation. Postgrad Med J 1979; 55: 303–310. PMID: 382161
28. Prentice AM, Roberts SB, Prentice A, Paul AA, Watkinson M, Watkinson AA, et al. Dietary supplementation of lactating Gambian women. I. Effect on breast-milk volume and quality. Hum Nutr Clin Nutr. 1983; 37: 53–64. PMID: 6341320

29. Marques AH, O'Connor TG, Roth C, Susser E, Bjørke-Monsen A. The influence of maternal prenatal and early childhood nutrition and maternal prenatal stress on offspring immune system development and neurodevelopmental disorders. Front Neurosci. 2013; 7: 120. https://doi.org/10.3389/fnins.2013.00120 PMID: 23914151

30. Ono KA, Boness DJ. The influence of El Nino on mother-pup behavior, pup ontogeny, and sex rations in the California sea lion. In: Trillmich F, Ono KA, editors. Pinnipeds and El Nino: Responses to Environmental Stress. Springer-Verlag, New York. 1991. pp. 185–192.

31. Zuk M, Stoehr AM. Immune defense and host life history. Am Nat. 2002; 160: Suppl 4:S9–S22. https://doi.org/10.1086/342131 PMID: 18707455

32. Rauw WM. Immune response from a resource allocation perspective. Front. Genet. 2014; 3: 267. https://doi.org/10.3389/fgene.2012.00267 PMID: 23413205

33. Kalnay E, Kanamitsu M, Kistler R, Collins W, Deaven D, Gandin L, et al. The NCEP/NCAR Reanalysis 40-year Project. Bull Amer Meteor Soc. 1996. 77. 437–471.

34. Peig J, Green AJ. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 2010; 118: 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

35. Inelmen EM, Sergi G. Biochemical Parameters of Nutrition. In: Mantovani G, Anker SD, Inui A, Morley JE, Rossi Fanelli R, Scevola D, Schister MW Yej SS, editors. Cachexia and Wasting: A Modern Approach. Springer-Verlag. 2006. pp. 59–72.

36. Bossart GD, Reidarson TH, Dierauf LA, Duffield DA. Clinical Pathology. In: Dierauf L, Gulland FM, editors. Marine Mammal Medicine. CRC Press, NY. 2001. pp. 383–436.

37. Hall AJ, McConnell BJ, Barker RJ. The effect of total immunoglobulin levels, mass and condition on the first-year survival of Grey Seal pups. Funct Ecol. 2002; 16: 462–474. https://doi.org/10.1046/j.1365-2435.2002.00649.x

38. Martin LB, Lewittes J, Kuhlman JR, Klasing KC Wikelski M. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol. 2006. 20. 290–299. https://doi.org/10.1111/j.1365-2435.2006.01094.x

39. Demas GE, Zysling DA, Beechler BR, Muehlenbein MP, French SS. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. J Anim Ecol. 2011; 80: 710–730. https://doi.org/10.1111/j.1365-2656.2011.01813.x PMID: 21401591

40. Vera-Massieu C, Brock PM, Godinez-Reyes CR, Acevedo-Whitehouse K. Activation of an inflammatory response is context-dependent during early development of the California sea lion. R Soc Open Sci. 2015; 2: 150108. http://dx.doi.org/10.1098/rsos.150108 PMID: 26064646

41. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2016. https://www.R-project.org

42. Norberg SE, Burkanov VN, Tuomi P, Andrews RD. Hematology of Free-Ranging, Lactating Northern Fur Seals, *Callorhinus ursinus*. J Wildl Dis. 2011; 47: 217–221. http://dx.doi.org/10.7589/0090-3558-47.1.217 PMID: 21270012

43. Kerr DS, Stevens MC, Robinson HM. Fasting metabolism in infants. I. Effect of severe undernutrition on energy and protein utilization. Metabolism 1978; 27: 411–435. PMID: 416321

44. Gulland FM, Hall AJ, Greig DJ, Frame ER, Colegrove KM, Booth RK, et al. Evaluation of circulating eosinophil count and adrenal gland function in California sea lions naturally exposed to domoic acid. J Am Vet Med Assoc. 2012; 241: 943–949. https://doi.org/10.2460/javma.241.7.943 PMID: 23013509

45. Maucher JM, Ramsdell JS. Domoic acid transfer to milk: evaluation of a potential route of neonatal exposure. Environ Health Perspect. 2005; 113: 461–464. https://doi.org/10.1289/ehp.7649 PMID: 15811837

46. Ramsdell JS, Zabka TS. In utero domoic acid toxicity: a fetal basis to adult disease in the California sea lion (*Zalophus californianus*). Mar Drugs. 2009; 6: 262–290. https://doi.org/10.3390/md20080013 PMID: 18728728

47. Cook P, Reichmuch C, Gulland F. Rapid behavioural diagnosis of domoic acid toxicosis in California sea lions. Biol Lett. 2011; 7: 536–538. https://doi.org/10.1098/rsbl.2011.0127 PMID: 21389016

48. Jeklova E, Leva L, Jaglic Z, Faldyna M. Dexamethasone-induced immunosuppression: a rabbit model. Vet Immunol Immunopathol. 2008; 122: 231–140. https://doi.org/10.1016/j.vetimm.2007.11.011 PMID: 18199487

49. Merlot E, Couret D, Otten W. Prenatal stress, fetal imprinting and immunity. Brain Behav Immunity. 2008; 22: 42–51. https://doi.org/10.1016/j.bbi.2007.05.007 PMID: 17716859

50. Honda T, Uehara T, Matsumoto G, Arai S, Sugano M. Neutrophil left shift and white blood cell count as markers of bacterial infection. Clin Chim Acta. 2016; 457: 46–53. https://doi.org/10.1016/j.cca.2016.03.017 PMID: 27034055

51. Lawler DF, Tangredi BP, Evans RH. Simultaneous hypo and hyper metabolic insults: effects on protein loss and survival in advanced malnutrition (Starvation). PMMC Case Rep Mar Mamm Path. 2014; 1: 1–8.

52. Westerman DA, Evans D, Metz J. Neutrophil hypersegmentation in iron deficiency anaemia: a case-control study. British J Haematol. 1999; 107: 512–515.

53. Palmer CS, Ostrowski M, Balderson B, Christian N, Crowe SM. Glucose metabolism regulates T cell activation, differentiation, and functions. Front Immunol. 2015; 6: 1. https://doi.org/10.3389/fimmu.2015.00001 PMID: 25657648

54. Mongini PKA, Stein KE, Paul WE. T cell regulation of IgG subclass antibody production in response to T-independent antigens. J Exp Med 1981; 153: 1–12. PMID: 6969777

ARTICULO III

En esta sección se presenta el tercer artículo derivado de la tesis, con título “Early hematological changes of free-living California sea lion pups (*Zalophus californianus*) as indicators of development, nutrition and health status”, que está en revisión en Journal of Wildlife Diseases.

CONCLUSIONES

Las células de la sangre participan en funciones vitales para el organismo, por lo que variaciones en el estado de salud, alimentación, y desarrollo pueden son reflejados en los elementos figurados de la sangre. Sin embargo, es importante contar con valores de referencia que permitan identificar alteraciones en cada componente celular hemático.

Para el lobo marino de California, antes de la realización de este trabajo, no existían valores hematológicos de crías en vida libre, por lo que se solían utilizar parámetros publicados para animales mantenidos en cautiverio o en rehabilitación, y por lo general se tomaban como referencia los valores de animales de mayor edad. En esta tesis se lograron establecer los valores de referencia para crías de lobo marino de California de vida libre a diferentes etapas de su primer año de vida, lo que fue esencial para identificar alteraciones en el estado de salud de los individuos. Durante el análisis de los valores hematológicos a lo largo del primer año de vida de las crías se pudo describir por primera vez en esta especie anemias fisiológicas; proceso que está bien documentado en humanos y animales domésticos. Aunque este tipo de procesos fisiológicos son resueltos por el individuo, puede indicar un momento crítico en el desarrollo de las crías que de no tener los elementos nutricionales adecuados pueden dificultar la resolución de dicho proceso.

También se observó que durante eventos climáticos atípicos y severos, como los ocurridos durante La Mancha y ENSO durante 2015, la modificación de los patrones de alimentación de las hembras adultas impacta a la fisiología sanguínea e inmune. Lo anterior significa que si las estrategias de alimentación de las hembras adultas son subóptimas entonces las crías tienen menos recursos energéticos para su desarrollo, por lo que procesos fisiológicos relevantes para las crías, como la síntesis y maduración de eritrocitos, esencial para una especie que bucea y la capacidad de respuesta inmune ante cualquier patógeno son impactados, probablemente con consecuencias severas para la población.

Específicamente se encontró que la prevalencia e intensidad de cambios morfológicos de los eritrocitos asociados con deficiencias nutricionales fueron mayores en crías en años anómalos con respecto a años con condiciones ambientales normales, además de que estas morfologías atípicas estuvieron influenciadas por la condición corporal y por las estrategias alimenticias de la madre. Además del impacto sobre los eritrocitos también se observó que durante años atípicos las crías exhibieron una menor condición corporal, una menor síntesis de inmunoglobulinas, así como una menor capacidad para responder de manera inespecífica ante desafíos inmunes. Esta es la primera vez que se estudia a detalle la morfología eritrocitaria de un mamífero marino, y que se demuestra que las morfologías atípicas, además de la restricción alimentaria que afecta las respuestas inmunes, están relacionadas con un evento climático. En el contexto de los cambios ambientales a los que se enfrenta el planeta, los resultados derivados de la tesis son importantes como evidencia de los efectos detrimentales del cambio climático.

LITERATURA CITADA

En esta sección se incluye la literatura que fue citada para la introducción de la tesis. Las referencias que se utilizaron para la elaboración de los tres manuscritos científicos están indicadas en los propios manuscritos.

Blaustein A., Belden L.K., Olson D.H., Green D.M., Root T.L., Kiesecker J.M. 2002. Amphibians breeding and climate change. *Conservation Biology*. 15(6) 1804-1809.

Boily, F., Beaudoin, S., Measures, L.N., 2006. Hematology and serum chemistry of harp (*Phoca groenlandica*) and hooded seals (*Cystophora cristata*) during the breeding season, in the Gulf of St. Lawrence, Canada. *J. Wildl. Dis*. 42, 115–132.

Bossart G.D. 2011. Marine Mammals as sentinel species for oceans and human health. *Vet. Pathol*. 48(3) 676-690.

Brock P.M., Hall A.J., Goodman S.J., Cruz M., Acevedo-Whitehouse K. 2013. Immune activity, body condition and human-associated environmental impacts in a wild marine mammal. *PLoS ONE* 8(6)e67132.

Clark, P., Boardman, W.S., Duignan, P.J., 2002. Cytology of haematological cells of otariid seals indigenous to Australasian waters. *Aust. Vet. J*. 80, 161–164.

Elorriaga-Verplancken F.R., Sierra-Rodríguez G.E., Rosales-Nanduca H., Acevedo-Whitehouse K., Sandoval-Sierra J. 2016. Impacto f the 2015 El Niño-Southern Oscillation on the abundance and foranging habits of Guadalupe fur seals and California sea lion from the San Benito Archipelago, Mexico. *PLoS ONE.* 11(5):e0155034

Fair, P. a., Hulsey, T.C., Varela, R. a., Goldstein, J.D., Adams, J., Zolman, E.S., Bossart, G.D., 2006. Hematology, Serum Chemistry, and Cytology Findings from Apparently Healthy Atlantic Bottlenose Dolphins (*Tursiops truncatus*) Inhabiting the Estuarine Waters of Charleston, South Carolina. *Aquat. Mamm.* 32, 182–195.

Föller M., Kaestner L., Straface E., Vogel J. 2011. Erythrocytes and Erythropoietin. *Int. J. Cell. Biol*. 2

France R.L. 1995. Carbon-13 enrichment in benthic compared to planktonic algae: foodweb implications. *Mar. Ecol. Prog. Ser.* 124:307–312.

García-Rodríguez F.J. and D. Aurioles-Gamboa. 2004. Spatial and temporal variation in the diet of the California sea lion (*Zalophus californianus*) in the Gulf of California, Mexico. Fish Bull 102:47–62.

Greig D.J., Gulland F.M.D., Rios C.A., Hall A. 2010. Hematology and serum chemistry in stranded and wild caught harbor seals in central California:

reference ranges, prognosis and explanatory factors. *J. Wildl. Dis.* 46(4), pp.110–113.

Hillman N., Kallapur S.G., Jobe A. 2012. Physiology of transition form intrauterine to extrauterine life. *Clin. Perinatol*. 39(4): 769-783.

Holinstat M. 2017. Normal platelet function. *Cancer Metastasis Rev*. 36(2): 195-198.

Jenssen, B. M. 2006. Endocrine-Disrupting Chemicals and Climate Change: A Worst-Case Combination for Arctic Marine Mammals and Seabirds? *Environ. Health Perspect.*, *114*(Suppl 1), 76–80.

Kintisch E. 2015. "The Blob" invades Pacific, flummoxing climate experts. *Science* 348:17–18.

Kling P.J., Schmidt R., Roberts R., Widness J.A. 1996. Serum erythropoietin levels during infancy: Associations with erythropoiesis. *J. Pediatr.* 128(6): 791-796.

Koury M.J., Ponka P. 2004. New insights into erythropoiesis: The roles of folates, vitamin B_{12}, and Iron. *Annu. Rev. Nutr.* 24: 105-131.

Kuhn V., Diederich L., Keller T.C.S. 4th., Kramer C.M., Lückstädt W., Panknin C., Suvorava T., Isakson B.E., Kelm M., Cortese-Krott M.M. 2017. Red blood cell function and dysfunction: redox regulation, nitric oxide metabolism, anemia. *Antioxid. & Redox Signal*. 1;26(13): 718-742.

Kuhn C.E. and D.P. Costa. 2014. Interannual variation in the at-sea behavior of California sea lions (*Zalophus californianus*). *Mar. Mamm. Sci.* 30:1297–1319.

Lombardero M., Kovacs K., Scheithauer B.W. 2011. Erythropoietin: A hormone with multiple functions. *Pathobiology*. 78:41-53.

Lowry M.S., B.S. Stewart, C.B. Heath, P.K. Yochem, and J.M. Francis. 1990. Seasonal and annual variability in the diet of California sea lions (*Zalophus californianus*) at San Nicolas Island, California, 1981–86. Fish Bull 89:331–336.

Michener R.H. and D.M. Schell. 1994. Stable isotope ratios as tracers in marine aquatic food webs. Pp. 138–157 in K. Lajtha and R.H. Michener, eds. Stable isotopes in ecology. Blackwell Scientific, New York.

Moore S.E. 2008. Marine mammals as ecosystem sentinels*. J. Mammal*. 89(3): 534-540.

Naigamwalla D.Z., Webb J.A. 2012. Iron deficiency anemia. *Can. Vet. J*. 53: 250-256.

Noren S.R., Poll C.P., Edwards M.S. 2018. Body growth rrapid hematological development support breath hold of baby belugas (Delphinapterus leucas) during subice transit. *Physiol. Biochem. Zool*. 91(1):691-704.

Nuñez L., Bouda J. 2007. Patología Clínica Veterinaria. 1era Edición. DR® Universidad Nacional Autónoma de México.

O'Brien R., Pearson H. 1971. Physiologic anemia of the newborn infant. *J. Pediatr.* 79(1): 132-138.

Parmesan C.2006. Ecological and Evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst*. 37:637-69.

Proytcheva M., A. 2009. Issues in neonatal cellular analysis. *Am. J. Clin. Pathol.* 131(4):560-73.

Rodak B.F. 2002. Hematología: Fundamentos y aplicaciones clínicas. Leucopoyesis. 2nd ed. Editorial Medica Panamericana.

Ruef P., Linderkamp O. 1999. Deformability and geometry of neonatal erythrocytes with irregular shapes. *Pediatr. Res*. 45, 114-119.

Skinner J P., Tuomi P A., Melllish Jo-Ann. 2015. The influence of time in captivity, food intake and acute trauma on blood analytes of juvenil Steller sea lions, *Eumetopias jubatus*. *Conservation Physiology*. 3, 1-12.

Tizard I.R. 2009. Introducción a la inmunología veterinaria. 8° edición. ELSEVIER.

Vera-Massieu C., Brock P.M., Godínez-Reyes C., Acevedo-Whitehouse K. 2015. Activation of an inflammatory response is context-dependent during early development of the California sea lion. *Royal Soc. Open Sci*. 2:150108