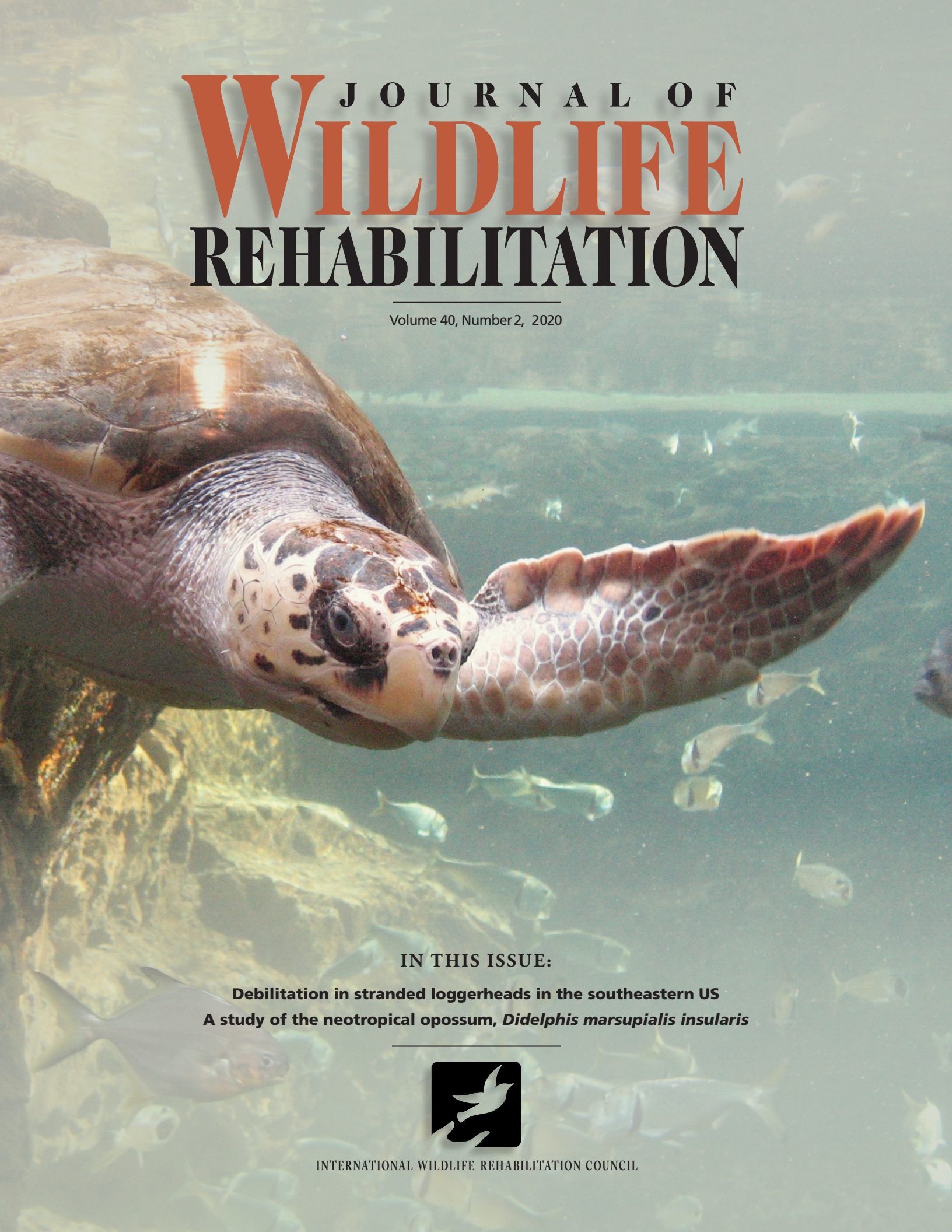
JOURNAL OF WILDLIFE REHABILITATION

Volume 40, Number 2, 2020



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Debilitation in stranded loggerheads in the southeastern US

A study of the neotropical opossum, *Didelphis marsupialis insularis*

INTERNATIONAL WILDLIFE REHABILITATION COUNCIL

THE *Journal of Wildlife Rehabilitation* is designed to provide useful information to wildlife rehabilitators and others involved in the care and treatment of native wild species with the ultimate purpose of returning them to the wild. The journal is published by the International Wildlife Rehabilitation Council (IWRC), which invites your comments on this issue. Through this publication, rehabilitation courses offered online and on-site in numerous locations, and its outreach to those in the profession, the IWRC works to disseminate information and improve the quality of the care provided to wildlife.

LEFT:
Island fox pup *(Urocyon littoralis)*. Four of the six Channel Islands subspecies were listed as Endangered in 2004.
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ON THE COVER:
Loggerhead turtle *(Caretta caretta)* detail.
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IWRC
International Wildlife Rehabilitation Council
PO Box 3197
Eugene, OR 97403 USA
Phone: 866.871.1869
Fax: 408.876.6153
Toll free: 866. 871.1869
Email: office@theiwrc.org
director@theiwrc.org
www.theiwrc.org

IWRC

Providing evidence-based education and resources on wildlife rehabilitation to move the field of wildlife rehabilitation forward; to promote wildlife conservation and welfare; and to mitigate human-wildlife conflicts worldwide, through better understanding of wild animal ecology, behavior, and welfare.

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EDITORIAL

Challenging Events Clarify Our Role

We are living through the days of a pandemic and it's interesting times for everyone, not the least wildlife rehabilitators. Challenges range from quarantine-limited staffing and the stress of succession planning (in case a permittee is infected with COVID-19), to heightened public interactions. And, of course, this pandemic has a suspected zoonotic origin. This leads us to ask: What role do wildlife rehabilitators play in preventing future pandemics?

The wildlife rehabilitation community called this play long before winter of 2020. We saw the story unfolding in our intakes, heard it coming in conversations with peers across the globe, and felt the frustration of unheeded scientists and veterinarians.

Fears of a new zoonotic pandemic, along with conservation, welfare, and scientific advancements have pushed us to acknowledge that the health of humans, our environment, and other species are linked—the One Health movement. It is time for us to double down and communicate science to government regulators and, more importantly, to the public that the elected officials of democratic nations answer to.

We need to not only provide raw facts, but tell the story of One Health, the many horrific stories of trafficking, the dangers lurking if we cannot coexist. Not scaremongering, not statistics, but stories. Rehabilitators play a part here—we, on the front lines of these issues, are a storehouse of experiences, and we have a direct line to the public.

The history of One Health is told not only in written words and curated images, but also through our policies, protocols, and procedures. These are in each phone call and text with the public. Our every interaction, intentional or not, tells a story. Through collaboration and the collection of these stories, I believe we can create a new narrative of respect, of biosecurity, of welfare first, of ecological integrity, of appreciation, of awe.

Our stories have the power to knit a better narrative of human–wildlife interactions. Step one: collect. Step two: share them with the world.

A Word on Racial Injustice

The International Wildlife Rehabilitation Council is a resource for wildlife rehabilitation knowledge. I've struggled with how to authentically address the issues of racism in the United States from IWRC's place as a source of rehabilitation information. I've found the answer right where I started; IWRC is a resource for wildlife rehabilitators, veterinarians, biologists, and others in the wildlife welfare and conservation fields. Skin color should not be a safety factor in rescuing an animal, and yet it is. We have the power to change that. Let's get to it. Gain knowledge; speak up: reflect on your actions, your reactions, even your confusion; and amplify the voices of our colleagues.

—Kai Williams
Executive Director

Birds Connect Our World

ITHACA, New York (May 9, 2020)—May 9th was World Migratory Bird Day. The 2020 theme was "Birds Connect Our World." The official day has passed but the World Migratory Bird Day organizations remind us we can celebrate any day of the year. Cornell has an excellent list of ways we can all support birds every day.

■ *Celebrate World Migratory Bird Day—Right at Home.* Check out online events and storytelling for kids, and find out how scientists learn where birds go. Visit the World Migratory Bird Day website.

■ *Make a Bird-Friendly Place in Your Yard or Neighborhood.* Provide shelter, nesting areas, and food for birds. You'll be amazed by the birds and other wildlife you can attract with a few simple steps. Learn about native plants and other ideas from Audubon.

■ *Watch Birds Near Home and Share What You See.* On May 9, participate in Global Big Day, and help set a record of 100,000 bird checklists submitted to eBird.org for use in science and conservation! Look out for the birds around your home, identify as many as you can, and share your list. Need help getting started? Download the free Merlin Bird ID app from the Cornell Lab of Ornithology or take the free eBird Essentials course.

■ *DIY Project: Make Your Windows Safer.* Up to 1 billion birds are estimated to die each year after hitting windows in the United States and Canada. Get the kids involved in a simple home improvement project to save birds by breaking up window reflections using string, decals, or paint spaced no more than two inches high or two inches wide. See quick, affordable ideas from American Bird Conservancy.

■ *Enjoy Indoor Time with Your Cat.* While outdoors, cats are estimated to kill more than 2.6 billion birds annually in the U.S. and Canada. Keeping your cat safe with quality time indoors also helps protect birds and other wildlife. See these six ways to keep your indoor cats happy.

PHOTO ©ROD WADDINGTON. CC BY-SA 2.0 LICENSE.

Gorilla beringei beringei, **Bwindi Inpenetrable Forest, a potential CoVid-19 victim?**

■ *Have a Bird-Friendly Drink.* Most coffee is grown in the sun, but shade-grown coffee preserves a forest canopy that helps migratory birds survive the winter, offers food and shelter for wildlife, and is economically and environmentally beneficial to farmers. Find out where to order Bird Friendly® coffee, certified by the Smithsonian Migratory Bird Center.

■ *Skip the Pesticides.* More than a billion pounds of pesticides are applied in the United States each year. Avoiding pesticides around your home and in your food is a healthy choice for wildlife and your family. Find out what produce contains the most pesticides.

■ *Reduce and Reuse Your Plastics.* It's estimated that 4,900 million metric tons of plastic trash have accumulated worldwide. Plastic waste is so pervasive that microplastics can be found in drinking water, and trash in the ocean entangles birds or is mistaken for food. Try these eight ways to reduce your plastic waste.

Wildlife Rehabilitation Centers Pivot to Operate Safely during COVID-19

EUGENE, Oregon, USA (May 5, 2020)—Multiple wildlife rehabilitation centers have seen financial and staffing challenges due to COVID-19; news reports include challenges for Prairie Wildlife Rehabilitation Center of Manitoba, MARS of British Columbia, Helping Hands Wildlife Center of Pennsylvania, Oak and Furrows of England, Wild Bird Rehabilitation and Wildlife Rescue Center in Missouri, and the New England Wildlife Center in Massachusetts.

Centers have been innovative in their response to the crisis, which came just before the northern hemisphere hit peak "baby season." The New England

Wildlife Center and Cape Wildlife Center converted their education vans into rescue vans. ED Katrina Bergman stated they felt it was no longer safe to have their doors open to the public, and going out on calls was the acceptable alternative. "Desperate times call for desperate measures," Bergman said. "We are an essential service and with a skeleton team already there, we now have two teams to respond to rescue calls in the education vans." Meanwhile their veterinary teams are sheltering in place at the clinics to care for wildlife.

The Wildlife Rescue Center in Ballwin, Missouri, has their volunteers working in small teams with non-overlapping shifts to minimize human to human contact. In California, Native Songbird Care & Conservation has a protocol that allows for contactless intake as does Dane County Humane Society's Wildlife Center in Wisconsin.

Other centers, like Minnesota's Wildwoods and California's Wildlife Rehabilitation & Release that are not able to intake wildlife at this time, or have very limited intake ability, are using their well-trained volunteers to provide phone support to the public.

In Florida, SPCA Tampa Bay, a longtime partner of Pinellas County rehabilitation centers has expanded its scope during the pandemic to directly rehabilitate wildlife.

COVID-19 a Threat to Great Apes

AFRICA (May 1, 2020)—While it is yet unknown if COVID-19 affects great apes, it's quite likely. Human respiratory viruses are an enormous threat. All across Africa governments have stopped tourism at great ape conservation sites. Additionally, teams in Uganda and the DRC are working with park rangers and local communities to encourage social distancing from great apes. The news magazine *Science* reports programs such as goat exchanges to reduce wild meat hunting during this critical time.

Global Call to End Wildlife Trafficking

HONG KONG (April 29, 2020)—As the world grapples with the worst global public health emergency in recent memory, more than 100 scientists and conservation leaders from 25 countries are calling on governments across the globe to address high-risk wildlife trade to reduce the chance of another outbreak.

In a joint letter to decision-makers, the experts note that COVID-19 is a zoonotic virus, meaning it was transferred from animals to humans, and that there is a real risk of future pandemics if no action is taken to reduce high-risk wildlife trade—especially in certain species of mammals and birds, which are more likely to host pathogens that can be transmitted to humans. High-risk situations where many animals, domestic and wild, dead and alive, from a variety of geographies, come into close proximity with one another and people in potentially unhygienic conditions pose a very high risk for disease spillover. These areas include markets, storage warehouses and transport hubs in densely populated areas.

More than 100 experts across nations, sectors, scientific disciplines and civil society agree that policy makers must take the following steps to reduce the chances of another pandemic:

- Shut down high-risk wildlife markets, with a priority focus on those in high-density urban areas.
- Urgently scale up efforts to combat wildlife trafficking and halt trade of high-risk taxa.
- Strengthen efforts to reduce consumer demand for high-risk wildlife products.

The letter brings together leaders in the fields of conservation, public health and zoonotic disease as part of the growing One Health movement that recognizes how our health is closely connected to the health of animals and our shared environment. Signatories include globally recognized One Health experts from the EcoHealth Alliance, the University of California-Davis, the Southeast Asia One Health University Alliance and Cornell University; the minister of health of Bhutan; a former secretary-general of the Convention on International Trade in Endangered Species (CITES); and leaders from the National Wildlife Federation, Wildlife Justice Commission and World Wildlife Fund (WWF).

Hon. Keith Martin MD, PC and Executive Director, Consortium of Universities for Global Health, Washington, DC, said:

> "We all need to speak out and engage elected officials to implement policies that will reduce demand for, and the trafficking in, endangered species; close those wildlife markets that have been identified to be high-risk conduits for disease transmission; and bolster the Global Health Security Agenda, which will strengthen the international community's capacity to prevent, detect and respond to disease outbreaks. Epidemics do not recognize borders, and neither can our response. Our health and safety depend on it."

The letter can be found in multiple languages on the website www.Prevent-Pandemics.org, and additional experts are invited to sign it through the website.

Partnership Spells Survival for Wiltshire Area Wildlife Centre

SWINDON, UK (April 24, 2020)—Oak and Furrows Wildlife Rescue Centre is partnering with RSPCA North Wiltshire to maintain operations. Times are challenging for small nonprofits. This English wildlife center has joined forces with their locally funded RSPCA as a way to maintain operations. In a Facebook post the organization noted,

> "All the staff at the centre are really excited for the future and how much more we will be able to do. We would like to extend a huge thank you to the local RSPCA for stepping in and keeping us going. Not only that, but another enormous thank you to all our lovely supporters and hope that you will join us in following our new wildlife rescue adventure!"

Now called RSPCA North Wiltshire Oak

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Chronic debilitation in stranded loggerhead sea turtles (*Caretta caretta*) in the southeastern United States: Morphometrics and clinicopathological findings

Nicole I. Stacy,[1] Jennifer M. Lynch,[2c] Michael D. Arendt,[3] Larisa Avens,[4] Joanne Braun McNeill,[4] Carolyn Cray,[5] Rusty D. Day,[2] Craig A. Harms,[6] A. Michelle Lee,[7a] Margie M. Peden-Adams,[7b] Kelly Thorvalson,[8] Al L. Segars,[3] and Terry M. Norton[9,10]

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ABSTRACT: Chronically debilitated loggerhead sea turtles *Caretta caretta* (DT) are characterized by emaciation, lethargy, and heavy barnacle coverage. Although histopathological findings associated with this condition have been reported, only limited data is available on health variables with clinical application. The objectives of this study were to 1) to compare morphometrics, clinicopathological variables, and immune functions of DTs to a group of apparently healthy loggerhead turtles to better understand the pathophysiology of the condition and 2) to assess health parameters in live debilitated turtles as they recovered during rehabilitation in order to identify potential prognostic indicators. We examined and sampled 43 DTs stranded from North Carolina to Florida for 47 health variables using standardized protocols to further characterize the condition. DTs were grouped into categories of severity of the condition, and those that survived were sampled at four time points through rehabilitation. All groups and time points were compared among DTs and to clinically healthy loggerhead turtles. Compared to healthy turtles, DTs had significantly lower body condition index, packed cell volume (PCV), total white blood cell (WBC) count, lymphocytes, glucose (Glc), total protein, all protein fractions as determined by electrophoresis, calcium (Ca), phosphorus (P), Ca:P ratio, potassium (K), lymphocyte proliferation, and greater heterophil toxicity and left-shifting, uric acid (UA), aspartate aminotransferase, creatine kinase, lysozyme,

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CORRESPONDING AUTHOR
Nicole I. Stacy
Department of Comparative, Diagnostic, and Population Medicine
College of Veterinary Medicine
University of Florida
Gainesville, FL 32601 USA
Email: stacyn@ufl.edu

Reprint: (2018). *PLoS ONE* 13(7):e0200355. https://doi.org/10.1371/journal.pone.0200355

Introduction

The loggerhead sea turtle (*Caretta caretta*) distinct population segment inhabiting the Northwest Atlantic Ocean is listed as threatened on the U.S. Endangered Species Act.[1] This population nests predominantly on Florida's Atlantic beaches, circumnavigates the Atlantic Ocean as young pelagic juveniles, and recruits back to U.S. coastal areas as older benthic-foraging juveniles. Benthic juvenile and adult loggerhead turtles are commonly observed in the stranding records along the southeast U.S. coast. Many of those found live are transported to rehabilitation centers where large resources of time and money are used for medical treatment with the objective to release them after successful recovery. Examination of stranded sea turtles can provide important information for understanding disease and stressors on sea turtle populations.[2,3] Common causes of stranding in sea turtles worldwide include watercraft or other traumatic injuries, drowning from fisheries interactions, hypothermia in winter months, and disease.[2,4–8]

For decades, chronically debilitated loggerhead turtles have been documented in the sea turtle stranding record along the southeast U.S. Informally called "barnacle bills," these debilitated turtles (DTs) present with emaciation, presence of numerous small barnacles covering the skin, and lethargy. Heavy epibiota can be normal on the carapace of healthy loggerhead turtles,[8,9] but they typically have only very low numbers of these commensals on the skin. Although specific cause(s) of chronic debilitation remain unknown, the current hypothesis is that this condition is consistent with the end stage of starvation and any cause preventing nutrient uptake or absorption can lead to this stage.[10] Comprehensive

health assessments, including physical examinations, detailed necropsies with histopathology, hematology, plasma chemistry panels, and other diagnostics can help elucidate any commonality and may point towards potential cause(s). Published studies on clinicopathological data of debilitated turtles are very limited to date. Deem et al.[8] examined health indicators in stranded versus foraging and nesting loggerhead turtles from Georgia. The stranded group, which included a mixture of DTs and turtles with various injuries (i.e., trauma, hook, neurologic abnormalities), had lower packed cell volume (PCV), total protein (TP), total solids (TS), monocytes, glucose (Glc), potassium (K), blood urea nitrogen (BUN), albumin (ALB), and globulins (GLOB); and higher lymphocytes, total white blood cells (WBC), creatine kinase (CK), and uric acid (UA) compared to foraging loggerhead turtles. These health indicators are consistent with anemia, reduced food intake, muscle injury, and wasting in the stranded turtle group. Similarly, stranded loggerhead turtles with a number of different stranding conditions in the Canary Islands were assessed for hematology and plasma chemistry.[11] The malnourished group (7 of 95 cases) in that study showed some similarities to the findings of Deem et al.[8] None of these reports were solely focused on chronically debilitated turtles, and a comprehensive evaluation of stranded debilitated turtles based on sampling of a broad suite of health variables during rehabilitation has been missing in the literature. Evaluation of data from turtles admitted from the same stranding cause has the potential to contribute to understanding the pathophysiology and may thus be helpful in the medical management of affected sea turtles during rehabilitation.

A perceived increase in DT strandings along the southeast U.S. prompted a workshop on St. Catherine's Island, Georgia, U.S.A in 2003 with experts of diverse specialties. The workshop resulted in a multi-organizational, multi-state (North Carolina, South Carolina, Georgia, and Florida) effort to conduct the current detailed investigation focused solely on DTs, both live and dead, using standardized collection protocols. Standardized protocols included a large number of measured variables: physical examination, morphometrics, genetic haplotype identification, epibiota identification, hematology, plasma biochemistry including testosterone for sexing, immune function, blood cultures, fecal parasitology, organic and inorganic contaminant tissue sampling, necropsy with parasite sampling and multi-organ tissue sampling for histopathology. Turtles were sampled through rehabilitation at four specified time points.

ABSTRACT, continued

and respiratory burst. From admission to recovery, hematology and plasma chemistry data improved as expected. The most informative prognostic indicators, as determined by correlations with a novel severity indicator (based on survival times), were plastron concavity, P, albumin, total solids, UA, lymphocyte proliferation, WBC, K, Glc, Ca:P, and PCV. The results of this study document the wide range and extent of morphometric and metabolic derangements in chronically debilitated turtles. Monitoring morphometrics and clinicopathological variables of these animals is essential for diagnosis, treatment, and prognosis during rehabilitation.

The objectives of this study were to 1) compare morphometrics, clinicopathological variables, and immune functions of DTs to a group of apparently healthy loggerhead turtles to better understand the pathophysiology of the condition and 2) assess health parameters in live debilitated turtles as they recovered during rehabilitation in order to identify potential prognostic indicators.

Materials and methods

Debilitated and healthy turtles sampled

Required federal and state permits were obtained for all activities in this study, including blood sampling. Permit numbers were National Marine Fisheries Service permits 1245, 1260, 1540, and 1551; South Carolina Department of Natural Resources MTP-2013-0005, Georgia Department of Natural Resources 1141, North Carolina Wildlife Resources Commission 01ST50, 02ST54, and 07ST53, and Florida Florida Fish and Wildlife Conservation Commission MTP12-021, MTP 140, and MTP 163. Chronically debilitated loggerhead turtles (DTs) sampled for this study stranded from July 2001 through June 2007 along the coast of North Carolina, South Carolina, Georgia, and Florida, U.S.A. Seventeen were found dead; 25 were found alive. Depending on the stranding location, live DTs were transported to rehabilitation facilities, including the Karen Beasley Sea Turtle Rescue and Rehabilitation Center, Topsail Island, North Carolina; South Carolina Aquarium, Charleston, South Carolina; Georgia Department of Natural Resources, Brunswick, Georgia and St. Catherines Island, Georgia; Volusia Marine Science Center, Ponce Inlet, Florida; Mote Marine Laboratory, Sarasota, Florida; and The Living Seas, Orlando, Florida. Dead chronically debilitated loggerhead sea turtles were necropsied at various locations; the necropsy data will be presented in a forthcoming publication.

Over 300 free-ranging, live captured, apparently healthy loggerhead turtles (HTs) of similar size, region, and collection years as DTs were selected as control turtles. Not all variables were measured on each HT; sample sizes for each analysis are reported in the Materials and Methods section or in Tables or Figures. HTs were captured by two long-term projects that maintain comprehensive databases of results. One project captured HTs in pound nets in Core Sound, North Carolina, through the NOAA National Marine Fisheries Service, Beaufort Laboratory. The second captured HTs in scientifically deployed trawl nets in coastal waters of South Carolina, Georgia, and northeastern Florida operated by the South Carolina Department of Natural Resources (SC DNR). All turtles captured by these two projects were measured and evaluated for abnormalities, such as emaciation, lethargy, injuries, and rehabilitation need. Abnormal findings are documented in the comments field of the databases. Turtles without comments related to debilitation or other abnormalities were selected as HTs. Life stage was determined based on straight carapace length (SCL). As size at maturity is variable and some loggerheads begin to nest around 75 cm SCL,[12,13] 80 cm SCL was selected as the cut off between neritic juveniles and adults.

TABLE 1. Sample sizes and characteristics of debilitated (DT) and healthy loggerhead turtles sampled in the current study. *Location and straight carapace length notch to notch (SCL n-n) percentages for healthy turtles were calculated on a representative 215 turtles. Statistically significant differences among DT categories are shown in brackets in the sample size row. https://doi.org/10.1371/journal.pone.0200355.t001

TURTLE GROUP	DT DEAD	DT DIED	DT SURVIVED				HEALTHY*
TIME POINT	N/A	A	A	B	C	D	N/A
TIMING OF SAMPLING	Upon dead stranding or after death	Upon live admission	Upon live admission	1 week after feeding on own	1 to 10 weeks after B, minor improvements	Upon release	Upon capture
SAMPLE GROUP	DT DEAD [X]	A–DIED [Y]	A-SURVIVED [A]	B	C	D	HT
N	18	13	12	≤7	≤6	≤9	>300
STRANDING YEARS	2004–2005	2002–2007	2001–2006				1998–2009
LOCATION (%):							
NC	17	23	42				63
SC	44	54	33				26
GA	28	15	25				9
FL	11	8	0				2
SCL (n-n) (%):							
<80 CM	83	85	75				88
>80 CM	17	15	25				12
SEVERITY INDICATOR (DAYS):							
Mean ± SD	0	6.23 ± 8.1	352.4 ± 123				N/A
Median (Range)	0 (0–0)	4 (1–31)	385 (47–463)				
N [sig dif from]	17 [YA]	13 [XA]	12 [XY]				

Physical examination and morphometrics

Each stranded DT included in this study was defined as a chronically debilitated turtle by visual observation of the following criteria: extensive epibiota coverage on the skin, emaciation based on sunken appearance in axillary, inguinal, plastron regions or eyes (S1 Fig.), lethargy, and no evidence of recent traumatic injury. Standardized digital photographs and measurements were taken, including straight carapace length (SCL) from the nuchal notch to the most posterior marginal notch, body depth, and body mass (S1 File). A body condition index was calculated as (mass in kg) divided by (cubed SCL in cm) and multiplied by 100,000.[14] A novel measurement of plastron concavity was also taken by resting a straight edge across the widest point of the plastron and measuring the distance from the bottom of the straight edge to the surface of the midpoint of the sunken plastron (S1 File). After completion of this study, veterinarians have concluded that plastron measurements using this method should not be performed on live DTs so as not to cause cardiac tears from sharp or broken plastron bones. Live DTs were further examined by veterinarians upon admission to rehabilitation facilities, where they received medical treatment based on the judgment of the responsible veterinarian.

DT health categories and sample collection time-points

Each DT was assigned to one of three health categories based on the individual's status at admission or their outcome (Table 1). "Dead DTs" (n = 18) were sampled post-mortem because they were found dead or died shortly after stranding either naturally or by euthanasia. Blood was collected from three dead DTs from the heart or dorsal cervical sinus within a maximum of four hours after the last eye reflex was observed. "A-died" (n = 13) turtles were sampled alive at time of stranding and were destined for a rehabilitation center but subsequently died. "A-survived" (n = 12) turtles successfully recovered in rehabilitation and were released. All samples collected from "A-survived" and "A-died" turtles were collected before or at time of admission to rehabilitation facilities before administration of any medications. During rehabilitation of "A-survived" turtles, additional sample collection time points were defined as the following: "B" = approximately one week after the sea turtle started eating independently; "C" = approximately one to ten weeks after the "B" sample, after minor improvements in body mass, body condition, behaviors, or clinical data, but before rehabilitation staff considered the turtle clinically recovered; and "D" = immediately prior to release (Table 1). It was not possible to collect all B, C, or D samples from all admitted DTs because of logistical constraints. Veterinary care and release criteria were determined by staff at the various rehabilitation centers.

Blood collection and handling

Blood sampling was part of a larger protocol that included collection of additional samples (e.g. parasitology, genetics) that will

be presented in a forthcoming publication (S1 File). Standardized protocols were followed for DT blood sample collection, handling, processing, and shipping to different laboratories. After sterile surgical preparation of the blood collection site, blood was collected from the dorsal cervical sinus using a 21-gauge 1.5 inch double-ended Vacutainer needle directly into several Vacutainers tubes (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.).[15] Several heparinized tubes were collected for hematology, plasma biochemistry, plasma protein electrophoresis, and immune function analysis at all four time points. Blood was also collected using sterile technique into bacterial blood culture medium (BBL® Septi-Chek,™ Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) at time points "A" and "D". The blood culture specimens were kept at room temperature and Vacutainer tubes cooled until processed. From one well-mixed heparinized whole blood tube, four blood films were prepared within 30 min, PCV was measured in-house by centrifuging capillary tubes at 12,000 × g for 5 min, and 0.3 mL of whole blood was transferred to a cryovial. The remainder of this and one other heparinized tube were centrifuged at 4,000 × g for 5 min. Plasma was transferred to several cryovials (Corning Incorporated, Corning, New York, U.S.A.) for plasma biochemistry (0.6 mL), bile acids and plasma electrophoresis (0.5 mL), biliverdin (0.2 mL), archiving (remainder), and in-house measurements of TS using a hand-held refractometer. The 0.6 mL plasma aliquot and 0.3 mL whole blood aliquot were shipped overnight on cold packs, with blood culture bottles well insulated away from cold packs, and processed by a commercial laboratory (Antech Diagnostic, Memphis, TN). The remainder of blood tubes and plasma aliquots and two blood films were transported same day or overnight on cold packs to Charleston, South Carolina, for processing immediately after arrival for immune function assays (Medical University of South Carolina) as well as sample archival and contaminant analysis at the National Institute of Standards and Technology (NIST) (mercury[16] and persistent organic pollutants [Lynch, personal communication]). Blood from HTs was collected and processed similar to DTs. Blood was drawn as quickly as possible after capture and generally processed within one to eight hours of collection for plasma chemistry and eight to 48 hours of collection for immune function assays.

Hematology

Blood films from DTs and a subset of 37 HTs were stained with Wright-Giemsa and blindly evaluated by one boarded clinical pathologist for WBC estimate, WBC differential (200 cells), and evaluation of blood cell morphology.[17] The WBC estimate was performed by multiplying the average number of WBC in 10 microscope fields × the objective power squared.[18] The erythroid regenerative response in anemic turtles was quantified by reporting the number of immature polychromatophilic erythrocytes per 100 mature erythrocytes as percentage (red blood cell [RBC] polychromasia %). In addition, subjective scores for RBC polychromasia, heterophil toxicity, and heterophil left-shift were used to characterize the degree by absent (0), mild (1), moderate (2), or marked (3).

Plasma chemistry panels and blood cultures

The following plasma biochemical analytes were measured at Antech Diagnostics for all DTs using a Beckman Olympus AU 5431 Chemistry Analyzer (Beckman Coulter Inc., Brea, California, U.S.A.): ALB (bromocresol green method), aspartate aminotransferase (AST), BUN, calcium (Ca), chloride (Cl), CK, GLOB, Glc, phosphorus (P), K, sodium (Na), TP (biuret method), and UA. Plasma from HTs were frozen at or below -70° C for typically five to ten days before analysis. Data from HTs were a mixture of unpublished data measured at Antech Diagnostics (Beckman Olympus AU 5431) or previously published data measured at North Carolina State University (Roche/Hitachi 912, Roche Diagnostics, Indianapolis, IN) as reported in Keller et al.[14] Blood culture results were reported by Antech at 14 days of incubation for DTs. No blood cultures were available from HTs.

Plasma protein electrophoresis, bile acids, and biliverdin

Plasma protein electrophoresis was performed at the University of Miami (Miami, FL) on all available DT plasma samples and 19 HTs using SPEP-II agarose gels and the Beckman paragon electrophoresis system (Beckman-Coulter Corporation, Brea, California, U.S.A.) with quantification of TP by biuret method (Kodak 750 X R, Ortho Clinical Diagnostics, Rochester, New York, U.S.A.). The gels were run as described previously.[19] The percentage of protein fractions was quantified by laser densitometry and then each fraction value was calculated by multiplying

the percentage of the fraction by the TP concentration. Bile acid analysis was performed at the University of Miami on all available DT plasma samples (no HT samples were analyzed) using the commercially available radioimmunoassay kit from MP Biomedicals (Solon, OH) as per recommendations by the manufacturer. The limit of detection was 0.1 μmol/L. Biliverdin concentrations were measured at the University of Georgia (Infectious Diseases Laboratory, Athens, GA) as previously described[20] on all available plasma samples from DTs and 20 HTs.

Immune function assays

Immediately upon arrival, a heparinized blood tube was centrifuged at 42 × g for 25 min to harvest peripheral blood leukocytes (PBLs) from the buffy coat for immune function assays at the Medical University of South Carolina. Mitogen-stimulated lymphocyte proliferation (LP) was measured on all available DTs as well as up to 77 HTs as previously described.[21,22] Briefly, viable PBLs were exposed to phytohemagglutinin P (PHA or lectin from red kidney bean (*Phaseolus vulgaris*); Sigma, St. Louis, MO, cat #L9132) and concanavalin A (ConA from jack bean [*Canavalia ensiformis*]; Sigma cat #C5275) to stimulate the T-lymphocyte proliferation, lipopolysaccharide (LPS from *Escherichia coli* 0111:B4; Sigma cat #L2630) to stimulate B-lymphocyte proliferation, and phorbol 12,13-dibutyrate (PDB; Sigma cat #P1269) to stimulate both T-and B-lymphocyte proliferation. The uptake of 3H-thymidine (ICN Biomedical, Irvine, CA) was measured from cells harvested onto Unifilter plates (Packard, Meridian, CT) using a Packard Top Count-NXT scintillation counter. The stimulation index (SI) is counts per minute (cpm) of mitogen-stimulated cells divided by the cpm of unstimulated control (media only) cells. The different incubation conditions are presented in the results as "mitogen_final mitogen concentration in the wells (μg/mL)_days of incubation" (e.g., PHA_5_5 means PHA at 5 μg/mL for five days).

Superoxide production, a measure of respiratory burst and thus innate immunity, was determined using PBLs by assessing nitroblue tetrazolium (NBT) conversion for all available DTs and 14 HTs. Briefly, 100 μL aliquots of PBLs diluted to 5 x 10^6 cells/mL in complete medium (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin and 50 μg streptomycin) were dispensed into 96-well plates containing triplicate wells of 60 μL of Ca ionphore (CI; 6.5 μL/ mL complete media solution made from a 1 mg/mL frozen stock in dimethyl sulfoxide [DMSO]), phorbol 12,13-dibutyrate (PDB; 3.1 μL/mL complete media solution made from a 1 mg/mL frozen stock in DMSO), or supplemented RPMI-1640 (unstimulated wells). To each well, 140 μg NBT (10 mg NBT in 56.8 mL of Hanks' Balanced Salt Solution with 2 mmol/L CaCl2) was added. Plates were incubated for 50 min at 30˚C and 5% CO2, then centrifuged at 1500 rpm (377 × g) for 3 min, and the supernatant was removed. KOH (120 μL of 2N) and 140 μL DMSO were added to each well, mixed by pipette, and plates were assessed for absorbance at 620 nm with a spectrophotometer (SpectraCount; Packard, Meridian, CT). The SI is absorbance units (AU) of stimulated cells divided by AU of unstimulated cells.

Plasma lysozyme activity, a measure of innate immunity, was determined using a standard turbidity assay described in Keller et al.[21] for all available DTs and 67 HTs.

Statistical analysis

We developed a novel method to numerically and objectively represent the severity of debilitation for each turtle as a continuum from one discreet health category to another (Fig. 1). We called this the severity indicator and used this continuous variable to assess correlations with clinicopathological variables most predictive of prognosis. We considered the order of severity from worst to least as 1) DT dead, 2) A-died that died shortly after admission, 3) A-died that died several days into rehabilitation, 4) A-survived that recovered slowly, and 5) A-survived that recovered quickly (Fig. 1). The duration of successful rehabilitation ranged from 47 days to 463 days, opportunistically providing an ideal large range for correlations. The severity indicator was calculated for A-survived turtles as the number of days between admission and a full recovery subtracted from 500 (an arbitrary value greater than the longest successful rehabilitation) so that the largest number represents the least severe turtle. For A-died turtles, the severity

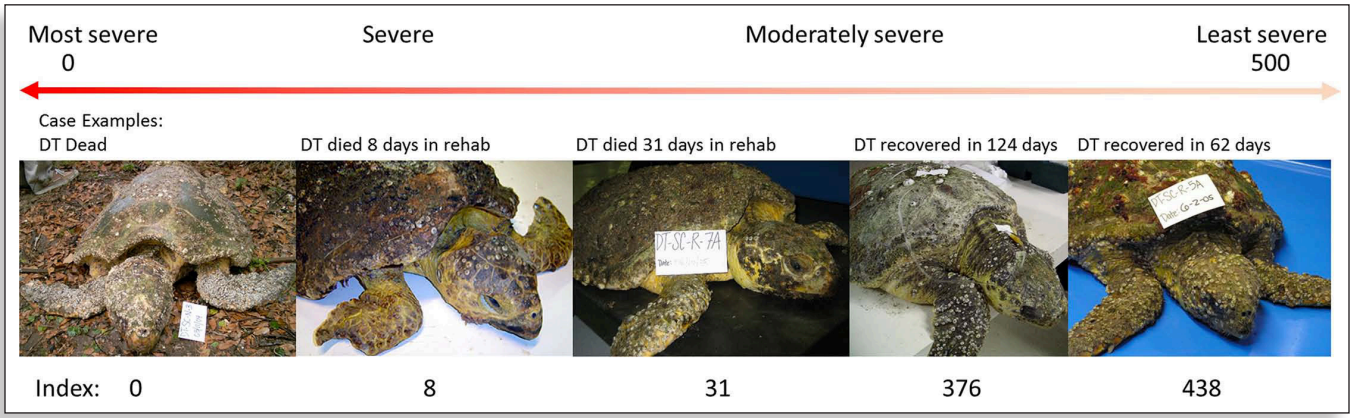

FIG 1. Diagram of severity indicator with five case examples of debilitated loggerhead sea turtles. Images show turtles at time of admission. https://doi.org/10.1371/journal.pone.0200355.g001

indicator was calculated as the number of days between the A sample and their death. Since those ranged from 1 to 31 days, again the larger the severity indicator, presumably the less severe the condition upon admission. DT dead were assigned the worst severity indicator of zero.

Statistical testing was performed using JMP 7.0.2 software (SAS, Cary, NC). Shapiro Wilk W and Bartlett tests were used to assess the distribution of the data and homoscedasticity, respectively. When either of these assumptions was not met, the data were log transformed. If data did not meet the assumptions, we chose the data (either raw or transformed) that were more normally distributed and more homoscedastic and checked statistical outcomes with non-parametric tests. To assess differences in health variables among A-survived, B, C, and D categories (e.g., improvement through rehabilitation), a repeated measures analysis of variance was used. If $p<0.05$, then paired t-tests were used to assess differences between each time point. Since these paired t-tests had to be performed individually (e.g. A-survived vs. B), a Bonferoni correction was used to determine significance ($p<0.00883$). To assess differences among all groups, including dead DTs, A-died, and HTs, which are independent turtles without repeated sampling, an analysis of variance was used including all health categories. If $p<0.05$, then a Tukey multiple comparison test was used to determine which health categories were different from these three independent health categories (e.g., do recovered turtles [D category] differ from HT?). Significance for the Tukey test was determined as $p<0.05$. Spearman correlations were performed for the severity indicator and all other health variables to investigate if more severely debilitated turtles had poorer health indicators and to determine which health variables were the most predictive of severity of the condition, thus providing an objective measure of prognosis. Values below the detection limit for bile acids were substituted with half the detection limit (0.05 µmol/L) for statistical testing. Because several samples had biliverdin concentrations below two different detection limits, statistical tests using the NADA package in R that appropriately handle left-censored data were used.[23] This also required a Bonferroni correction of alpha due to the 21 pairwise comparisons made ($p<0.00238$ was considered significant when using this test).

To investigate methodology differences in TP (biuret method) vs TS (refractometry) and ALB by bromocresol green method vs protein electrophoresis, Spearman correlations and paired t-tests were performed. Comparisons among TP, ALB, and TS included data only from DTs (all DT categories and time points were combined).

Results and discussion

Characteristics of sampled DTs

A total of 43 live and dead DTs from North Carolina, South Carolina, Georgia, and Florida were included in this study (Table 1). Of the turtles admitted alive (n = 25), 13 (52%) died. The "A-died" category included ten turtles that died within eight days after admission, one that died at day 31, and two that were

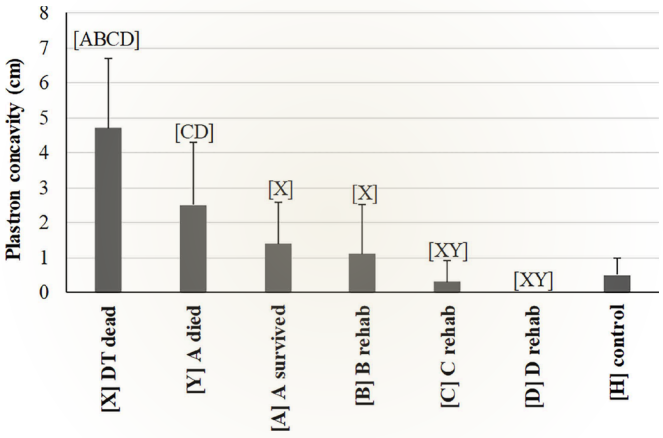

FIG 2. Comparison of plastron concavity measured in debilitated loggerhead sea turtles (DTs) before and during rehabilitation and compared to healthy loggerhead turtles (means and one standard deviation). Lines represent an individual turtle through rehabilitation. Each turtle group on the x-axis was significantly different ($p<0.05$ or $p<0.00883$ for repeated measures) from groups represented by letters above data. Sample size for each group: 15 [X], 9 [Y], 6 [A]–[D], 1[H]. https://doi.org/10.1371/journal.pone.0200355.g002

euthanized on the day of admission. These data highlight the high mortality rate and guarded prognosis for DTs. The DTs of the category "A-survived" had an average rehabilitation time of 148 days (range 47 to 463 days). For live turtles during rehabilitation, samples were collected at time point "B" within 6 to 32 days after admission, "C" within 14 to 80 days after admission and "D" within 47 to 463 days after admission. In comparison, the turtles in the current study were in rehabilitation longer (mean = 148 days) than loggerhead turtles that stranded due to malnutrition in Gran Canaria Island, Spain (median = 43 days).[24] This suggests that loggerheads in the Spanish study likely represent a less severe degree of emaciation than compared to DTs of our study.

Most DTs sampled in this study stranded in SC (Table 1). This was an artifact of sampling effort being strongest in SC and should not be considered typical for spatial stranding trends of DTs in the southeast U.S. Approximately 80% of the DTs were categorized as neritic juveniles (<80 cm SCL) and 20% were adults. Seasonally, stranded DTs included in this study peaked between May and July (n = 4 in April, 17 in May, 9 in June, 6 in July, 3 in August, 0 in September, and 4 in October). Since season influences the immune system of reptiles,[25] it is important to note that immunology was performed on HTs captured only in the summer months, coinciding with the peak season of the DT stranding.

The severity indicator (a measurement of the number of days to death or recovery) significantly decreased with increasing severity across the three DT categories as expected (Table 1). Morphometric data and significant differences among all seven health categories of turtles are summarized in S1 Table. SCL did not differ among the seven categories which suggested turtles of similar sizes and age classes were being compared. Body mass improved from time points A and B to time point D during rehabilitation as expected. Our novel method for quantitatively

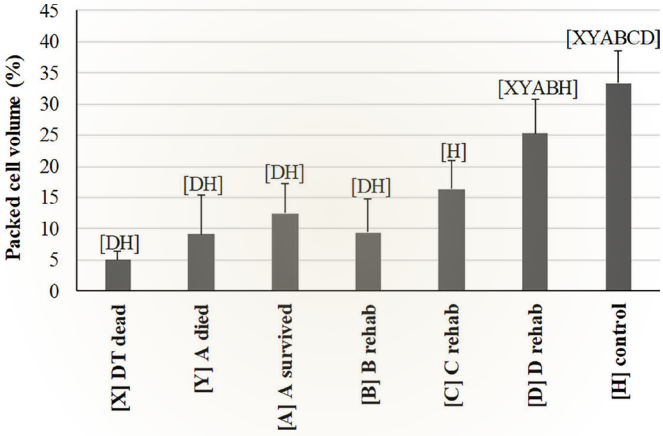

FIG 3. Comparison of packed cell volume (PCV) measured in debilitated loggerhead sea turtles (DTs) before and during rehabilitation and compared to healthy loggerhead turtles (means and one standard deviation). Lines represent an individual turtle through rehabilitation. Each turtle group on the x-axis was significantly different ($p<0.05$ or $p<0.00883$ for repeated measures) from groups represented by letters above data. Sample size for each group: 2 [X], 10 [Y], 11 [A], 7 [B], 6 [C], 9 [D], 69 [H]. https://doi.org/10.1371/journal.pone.0200355.g003

measuring plastron concavity (S1 File) proved to show expected trends (Fig. 2): it was deepest in DT dead (deeper compared to all other categories) and A-died (deeper than C, D) with steady improvement through rehabilitation to a complete lack of concavity by the time of release. It is important to note that this measurement is not recommended in live DTs due to the high risk of cardiac tears from plastron bones when turning DTs onto their backs. Body condition index was lower in DT dead, A-died, and A-survived compared to D and HTs, whereas the index at time points B and C were lower only compared to HTs (S1 Table). These findings confirm the extent of emaciation that defines DTs during visual and physical examination. The significant improvements of body mass, plastron concavity, and body condition index by time point D during rehabilitation are consistent with the reversal from a catabolic to an anabolic state and restoration of energy stores. This is the first study to present these improvements specifically in debilitated loggerhead turtles.

Blood culture

Blood cultures for 15 DTs ("A-survived" n = 6; "A-died" n = 7; "DT dead" n = 2) were collected at time of admission. Five samples were positive; one microorganism was found in each of the positive samples: *Aeromonas hydrophila/caviae* (A-survived), *Pseudomonas putida* (A-died), *Vibrio parahemolyticus* (A-died), *Shewanella putrifaciens* (dead DT), *Shewanella algae* (dead DT). The *Shewanella* sp. cultured from the dead DTs could have resulted from post-mortem contamination, while the other organisms may have pathogenic significance in a clinically sick turtle. All blood cultures collected at time point D (n = 4) were negative, likely as the result from treatment and immune response.

Hematology and morphological evaluation of blood films

Hematology results and significant differences among the seven turtle categories are summarized in S2 Table. Significant differences in one or more of three DT categories at the time of admission (Dead DT, A-died, or A-survived) compared to HTs included lower PCV, total WBC, heterophils, lymphocytes, and monocytes and higher degrees of heterophil toxicity and left-shifting. The lower PCV, indicating anemia, was consistent with stranded turtles from Georgia in Deem et al.[8] and the malnourished group from the Canary Islands in Casal and Oros.[11] This similarity across studies indicates that PCV is a clinically important variable when examining sick loggerhead turtles. Deem et al.[8] also found lower monocytes, but no change in total WBC or heterophils and an opposite trend (elevation) for lymphocytes. Casal and Oros[11] observed a similar decrease in total WBC and lymphocytes, but opposite trends for heterophils and monocytes. The differences among studies in cell counts could be due to different causes for stranding or emaciation, different stages of disease progression, or differences in methodology.

During rehabilitation, between A-survived and time point B, PCV, and lymphocytes decreased, and heterophil:lymphocyte ratio, and heterophil toxicity and left shifting increased (S2 Table). This indicates that during this initial stage of supportive care, these blood analytes get worse before they begin to improve. This is undoubtedly a very active phase in which supportive nutritional care and antimicrobial administration work together to mount significant and diverse physiological changes to reverse catabolism. Rehydration may cause PCV to decrease initially until hematopoietic tissues respond, notably to the anemia. Nutrition appears to provide the energy needed for hematopoietic and lymphoid tissues to mount effective immune responses.

PCV (Fig. 3) was significantly and dramatically lower in DT dead, A-died, A-survived, and B compared to D and HT. During rehabilitation, PCV dropped at B likely in response to fluid therapy and rehydration, significantly improved by D, but never recovered to ranges of HTs. The only other hematological variable that significantly differed between A-survived and D was an increase in RBC polychromasia evaluated as by score and percentage (S2 Table). Eosinophils and basophils showed little to no differences between DTs and HTs or between A-survived and D. These findings are similar to Harms et al.,[26] in which PCV increased and no change in WBC counts or the leukocyte differential counts were observed in loggerheads undergoing rehabilitation after stranding from various causes not including debilitation. No blood parasites or other infectious agents were observed on any blood film.

The morphological evaluation of RBC polychromasia is critical in the identification of erythroid regeneration in anemic reptiles. Given the lack of standardization in the quantification of the erythroid regenerative response in non-mammalian vertebrates, two approaches were attempted in this study. Both a subjective RBC polychromasia score and a numerical RBC polychromasia percentage provided similar results with concurrent improvement in PCV and a peak in RBC polychromasia by both methods at time point C (S2 Table). At stranding, eight

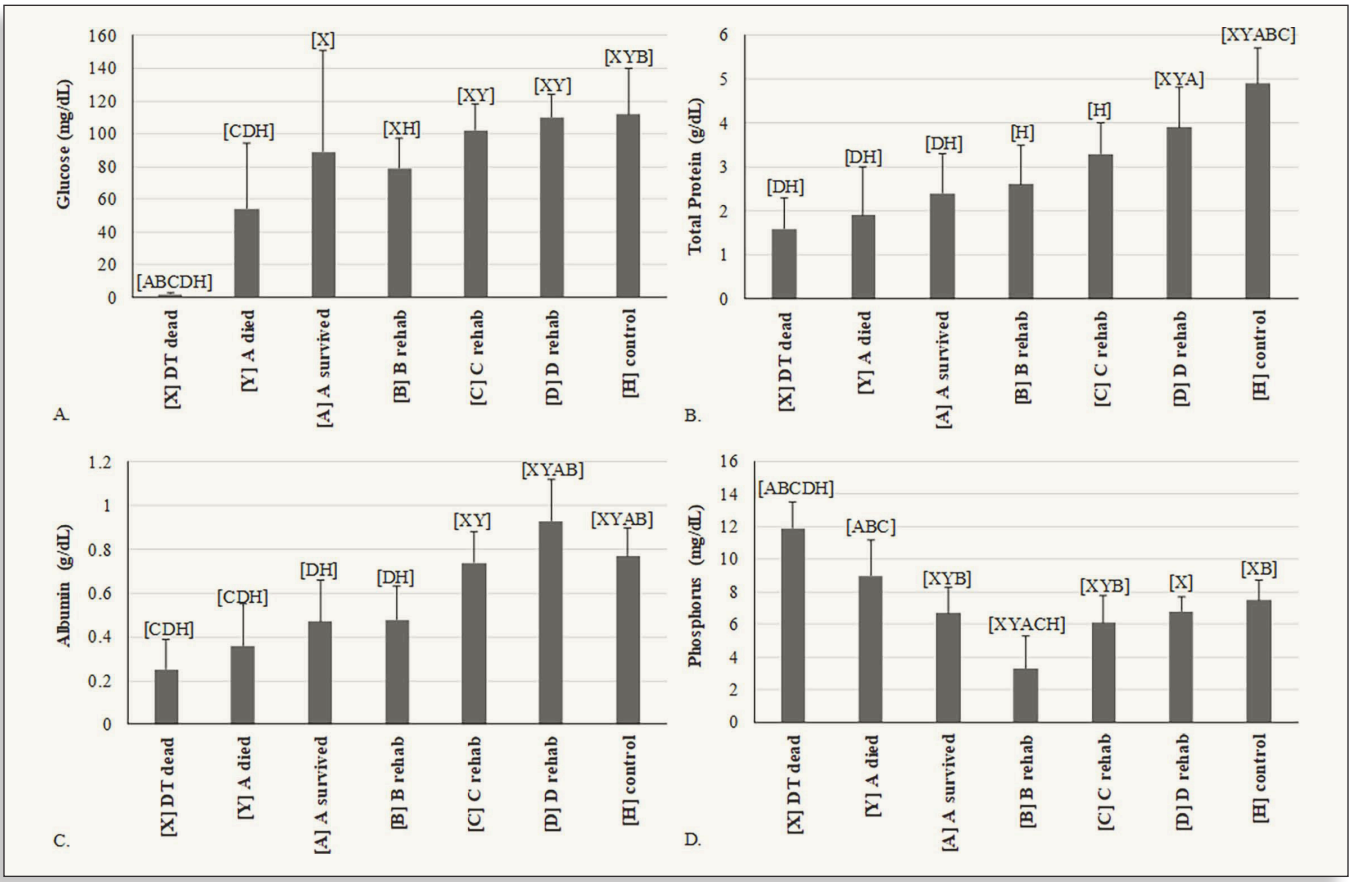

FIG 4. Comparison of variables measured in debilitated loggerhead sea turtles (DTs) before and during rehabilitation and compared to healthy loggerhead turtles (means and one standard deviation). Each turtle group on the x-axis was significantly different ($p<0.05$ or $p<0.00883$ for repeated measures) from groups represented by letters above data. (A) glucose; (B) total protein by biuret method at U. Miami; (C) albumin by plasma electrophoresis; (D) phosphorus. Lines represent an individual turtle through rehabilitation. For samples sizes in each diagram, please refer to S3 Table. https://doi.org/10.1371/journal.pone.0200355.g004

out of 12 DTs had severe, non-regenerative anemia (PCV <12%, RBC polychromasia score = zero). The remaining four DTs had moderate, non-regenerative anemia (PCV = 14% to 21%, RBC polychromasia score = zero). We investigated the time it took to observe the first evidence of erythroid regeneration in the DTs that were followed through rehabilitation using the criteria of PCV rising above 14% and RBC polychromasia scores becoming at least one. This was observed at 56 days after admission (mean; range 24–108 days) occurring in time points B or C. But it took longer before the PCV increased to a minimum of ≥ 20%, which occurred by day 103 (mean; range 36–148 days) at time points C or D for all turtles that were closely followed using complete sets of blood films for all time points. However, in some turtles, no overt erythroid regeneration (e.g., polychromasia) was observed at the defined time points during rehabilitation despite resolving anemia. Lack of an observed erythroid response by blood film evaluation may have been due to variable frequency of sampling during recovery. Had the study been extended, we may have observed a morphologically recognizable erythroid response. The clinical importance of the long duration to full recovery of PCV underlines the severity of the condition and the duration of supportive care needed in order to elicit a response by hematopoietic tissues to the chronic anemia.

The degree of the anemia and slow recovery of PCV in the admitted DTs reflects the severity of debilitation. The complexity of the disorder and end-stage presentation of the patients precludes identification of specific cause(s) of anemia; however, the causes are likely multifactorial and difficult if not impossible to identify as previously reported for a stranded group of loggerhead turtles.[8] Considerations for the causes of anemia in DTs include bone marrow suppression and reduced erythropoiesis from malnutrition, inflammatory and/or chronic disease(s), and/or bacterial or parasitic infections, among other ailments seen in the DTs. Regardless from which cause(s) the anemia initially resulted, the clinical significance of anemia lies in the reduced oxygen delivery to tissues, which may lead to further organ compromise from hypoxia, especially in highly vascularized tissues such as liver, kidneys, and lungs.

Leukocyte morphological changes can be useful prognostic indicators in mammals.[27] Mild to marked heterophil toxicity was observed in ten of 17 DTs at presentation or time of admission and heterophil left-shifting in 13 of these DTs. Low numbers of melanomacrophages (<5/blood film) were noted in two A-died, one A-survived and throughout its rehabilitation, and at the D

time point for two additional A-survived turtles. Heterophil toxicity reached a score of zero by day 49 (mean; range 8–124; time points B or D), and heterophil left-shifting took longer to disappear with 95 days into rehabilitation (mean; range 56–151; time point D). The leukogram findings, especially the frequency and duration of heterophil left-shifting, indicate ongoing active systemic inflammation in the DTs. In mammals, neutrophil toxicity is considered non-specific and can be seen in various disorders, including systemic inflammation, bacterial or other infections, tissue necrosis, and some severe metabolic disorders.[27] In dogs, the presence of these cytoplasmic features of toxicity is associated with higher mortality rates, and their disappearance correlates well with clinical improvement, thus offering a prognostic indicator.[27] In reptiles, heterophil toxicity appears to have similar prognostic significance as well as heterophil left-shifting.[17] Left-shifting is defined as the presence of immature heterophils, which indicates a need for active production of these inflammatory cells by hematopoietic tissues in response to increased tissue demand. The data on these DTs support the usefulness of these morphological changes as diagnostic indicators and for monitoring. Moreover, melanomacrophages can be a rare finding in healthy reptiles, but an increased number circulating in ill reptilian patients may indicate chronic inflammation/disease, cachexia, or other nonspecific conditions.[28,29]

Plasma chemistry

Mild hemolysis was present in one of three dead DTs, and plasma was green for three turtles sampled at admission (two A-survived and one A-died). Green plasma has been associated with starvation, liver disease, and hemolytic disease in sea turtles[29] and it is unknown how the green discoloration may affect the analysis of various chemistry analytes. Plasma biochemistry results and significant differences among the seven turtle categories are summarized in S3 Table. Severe metabolic derangements were observed in the three DT categories at the time of admission (Dead DT, A-died, or A-survived). Significant differences in one or more of those categories compared to HTs included decreased Glc, TP, all protein fractions, Ca, Ca:P ratio, and K and increased P, Na, Cl, UA, AST and CK. The reduced Glc and protein concentrations are consistent with other studies that examined stranded loggerhead turtles.[8,11,30] Deem et al.[8] also observed the decrease in K and increases in UA and CK in the stranded group, but not the changes we noted in other electrolytes or AST. The stranded turtles in the Canary Islands showed the opposite, lower UA and AST compared to the DTs in the current study.[11] These comparisons suggest that lack of nutrition (decreased Glc and TP) from anorexia is present in most stranded turtles, regardless of the cause of stranding, and the changes in electrolytes and AST seem more common in turtles with chronic debilitation.

During rehabilitation, most variables improved as expected (S3 Table). Notable trends were observed for Glc, TP, ALB, and P (Fig. 4). Interestingly, between A-survived and time point B, several variables, including Glc, Ca, K, AST, and CK, tended to worsen (S3 Table). This observation suggests that even with early treatment, plasma chemistry indicators of debilitation tend to worsen before improvement as critical physiologic changes occur during this initial phase reversing the effects of starvation. This mirrors the above discussed hematological finding suggestive of an inflammatory response after treatment initiation at time point B: the chemistry findings may indicate a stimulation of internal organs in response to supportive care in the effort to correct metabolic derangements after an extended time of anorexia and organ compromise. Other variables with statistically significant changes between A-survived and D include increases in alpha 1-, and beta-globulins, BUN, and K, and a decrease in AST (S3 Table). Variables that were not as severely deranged (e.g., showed little to no differences between DTs and HTs or between A-survived and D) were TS, ALB:GLOB ratio, bile acids, and biliverdin. Three other studies have followed plasma chemistry values through rehabilitation of loggerhead sea turtles.[4,26,31] The increase in BUN was consistently noted in all three studies, but none of the studies saw an increase in Glc, since only few turtles initially presented with hypoglycemia. The increases in TP, ALB, and GLOB were also observed in Harms et al.[26] The increase in K was seen by Harms et al.[26] but not by Camacho et al.[31] Harms et al.[26] observed an increase in P and no change in AST, which are different from the current study.

Glc, similar to PCV, dropped between A-survived and B with subsequent improvement by C to healthy ranges (Fig. 4A). Hypoglycemia has been documented in stranded loggerhead turtles,[8] especially those with malnutrition.[11,32] Although the extremely low Glc concentrations in DT dead (≤ 2 mg/dL) may have been falsely decreased by prolonged contact of the plasma with erythrocytes in vivo, this artifact was likely mild, since all three blood samples were collected within four hours after death and immediately processed; additionally, this degree of severe hypoglycemia was also observed in a live DT upon admission. Based on our results, hypoglycemia in DTs may be associated with hyporexia or anorexia, exhaustion, exertion, malnutrition, decreased gluconeogenesis, and/or sepsis. One A-survived turtle had moderate hyperglycemia (244 mg/dL) at admission, which may be explained by metabolic stress or stress of stranding and/or handling in this individual. The Glc fell to 68 mg/dL 4 days after admission (B) in this individual turtle, and normalized by 41 and 62 days after admission (C and D, respectively) to between 115 and 111 mg/dL.

TP (Fig. 4B) and ALB (Fig. 4C) were lowest at admission of DTs but increased to ranges similar to HTs by D or C, respectively. All three groups of DTs were severely panhypoproteinemic at time of admission, with significant, steady improvement during rehabilitation and recovery to concentrations comparable to healthy turtles by time point D (S3 Table). Previously concluded causes for panhypoproteinemia in stranded loggerhead turtles include malnutrition, parasites, and protein-losing disorders.[8] The lower ALB: GLOB ratio in non-survivors is consistent with significantly lower ALB concentrations, with considerations including condi-

tions with decreased ALB synthesis (e.g., inflammation, malabsorption/maldigestion, hepatic insufficiency) or albumin loss (e.g. through gastrointestinal tract, kidneys, skin, or blood loss). Pre-albumin, which includes thyroid-hormone-binding protein (transthyretin) and vitaminbinding proteins in other species,[33,34] as well as albumin, alpha-1-, alpha-2-, beta-, and gamma-globulins all demonstrated steady improvement during rehabilitation with ranges at recovery at or approaching that of HTs (S3 Table). Most protein fractions decreased between time points A and B, which tracks with PCV, and may be explained by rehydration and hemodynamic improvement from fluid treatment.

It was important to compare the different measures and methods of protein (TP, ALB, TS) to determine if methods substantially deviated from each other. The three investigated correlations were statistically significant ($p<0.0001$) with positive slopes, suggesting that generally each variable or method tracks the other, but they all fell below the 1:1 line: TP by biuret method [Antech] vs. TS (n = 24); TP by biuret method [U. Miami] vs. TS (n = 21); and ALB by bromocresol green method [Antech] vs. ALB by protein electrophoresis [U. Miami] (n = 41) (n = 41; S2 Fig.). ALB by bromocresol green method [Antech] was significantly greater than ALB by protein electrophoresis [U. Miami] by 0.17 g/dL on average (S3 Fig.). TP by biuret method measured at Antech and U. Miami were on average 0.34 and 0.41 units higher than TS measured by refractometry in-house, respectively. These differences were statistically significant (S3 Fig.), and could be clinically relevant especially for hypoproteinemic turtles. TS are frequently performed in-house to estimate TP but are affected by increased concentrations of osmotically active analytes, such as Glc, Na, and BUN. The extremely low TS in DTs are primarily because the DTs had low TP but may also be caused in part by the very low Glc and BUN concentrations or inaccuracies of reading the refractometer at such low TP concentrations. The significantly greater ALB by bromocresol green method compared to ALB by protein electrophoresis in DTs is consistent with a recent report in diseased Hermann's tortoises.[35] This is likely due to assay interference of substrates other than ALB, such as fibrinogen or globulins, in the bromocresol green method or inaccuracies at very low ALB concentrations.[34,35] Similar to other reports, we emphasize the importance to consider these limitations of the bromocresol green method and to utilize protein electrophoresis if accurate ALB concentrations are desired in sea turtles.

The BUN concentrations demonstrated an increase through rehabilitation (D was greater than A-survived) to a degree that

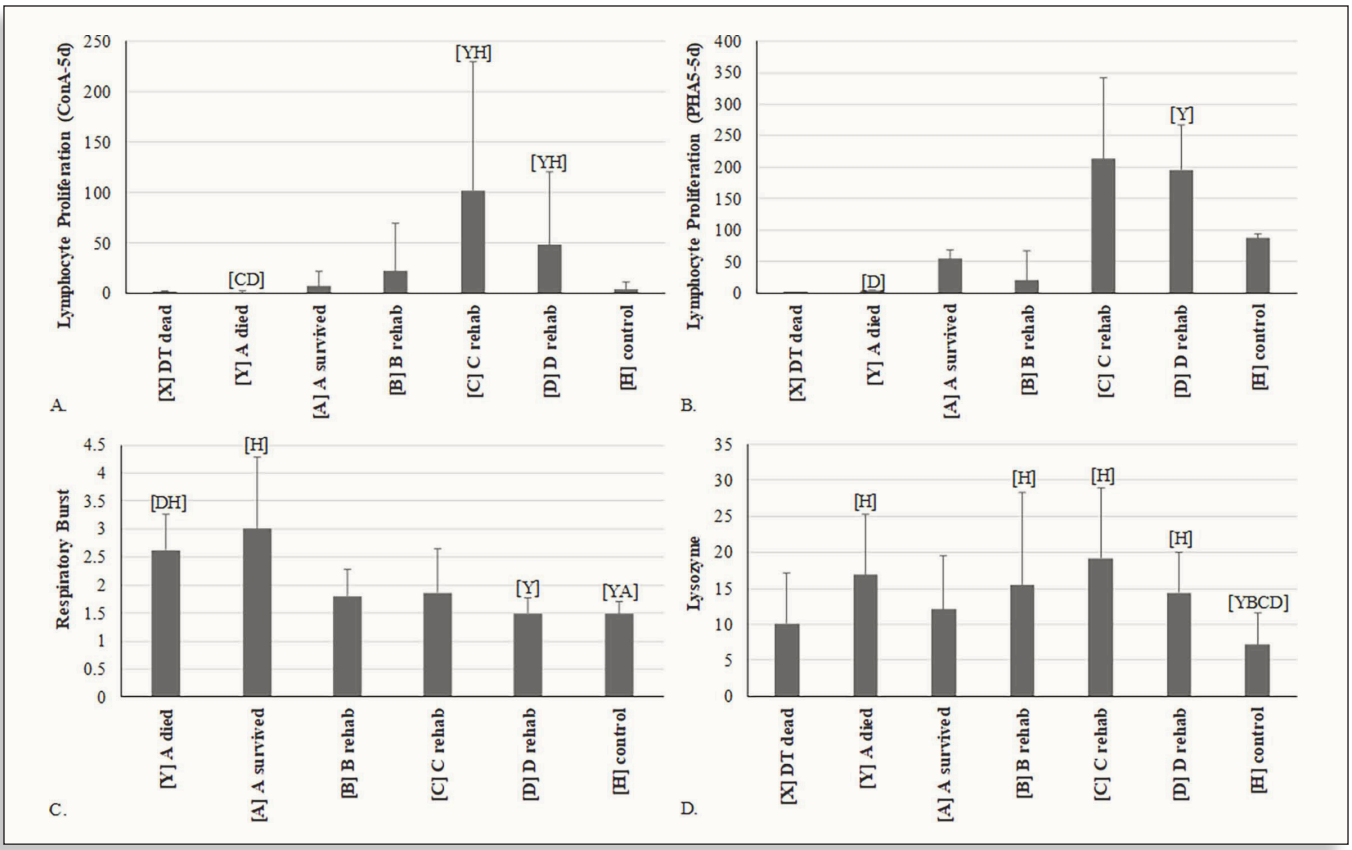

FIG 5. Comparison of immune function variables measured in debilitated loggerhead sea turtles (DTs) before and during rehabilitation and compared to healthy loggerhead turtles (means and one standard deviation). Each turtle group on the x-axis was significantly different ($p<0.05$ or $p<0.00883$ for repeated measures) from groups represented by letters above data. (A) lymphocyte proliferation with ConA; (B) lymphocyte proliferation with PHA; (C) respiratory burst with Ca ionophore; and (D) lysozyme activity. Lines represent an individual turtle through rehabilitation. For samples sizes in each diagram, please refer to S4 Table. https://doi.org/10.1371/journal.pone.0200355.g005

was significantly higher compared to HTs (S3 Table). Low initial BUN likely reflects chronic starvation and possibly hepatic insufficiency, while production of BUN increases through rehabilitation after acceptance of food and/or recovery of liver function after hemodynamic improvements from treatment and rehydration.[6] Higher BUN in rehabilitated DTs compared with HTs may reflect consumption of a higher protein and higher quantity diet by turtles in captivity compared with free ranging HTs. BUN concentrations higher than healthy free-ranging turtles have been seen in captive rehabilitated stranded loggerhead sea turtles[26] and convalescent cold-stunned Kemp's ridley sea turtles.[6]

The Ca: P ratio was lower in the DTs that died and significantly increased at time point B compared to HTs (S3 Table). P appeared to drive these differences more than Ca, as Ca was only mildly low at time of admission and B. P (Fig. 4D) was highest in DT dead and A-died. It was observed at concentrations similar to HTs in A-survived, C and D, but a significant decrease was seen at B. In fact, P decreased without a concurrent Ca decrease, resulting in marked hypophosphatemia. This observation may be explained by increased urinary excretion after hemodynamic adjustments from rehydration/fluid therapy, and/or prolonged intestinal malabsorption or anorexia at time point B. Ca:P is used for diagnosis of renal disease in terrestrial reptiles,[36] but caution is warranted in using this ratio in marine turtles. Ca and P homeostasis is often reversed and can be significantly influenced by dietary availability and bone growth in sea turtles.[6] Various factors need to be considered when interpreting this ratio in DTs. Hypoalbuminemia likely contributes to reduced protein-bound Ca fractions in plasma of DTs that died. It is ideal to use ionized Ca measurements for the assessment of the biologically available Ca concentrations,[37] which were not performed in this study. Nutritional deficiencies or differences (wild vs. captive diet) may also contribute to hypocalcemia in DTs. High P concentrations at the time of stranding could be associated with reduced renal blood flow and decreased renal P excretion given the evidence of dehydration in DTs (visible sunken eyes, hyperuricemia). Muscle injury or wasting may also contribute to higher plasma P.

K was significantly higher in dead DTs and lower in all other DTs than HTs with some improvement between time points A and D (S3 Table). High K concentrations in dead DTs were possibly affected by post-mortem leakage of K from tissues in prolonged contact with plasma or from difficult blood withdrawal (this information was not recorded). Additional considerations include presence of metabolic acidosis, muscle injury, and/or decreased renal

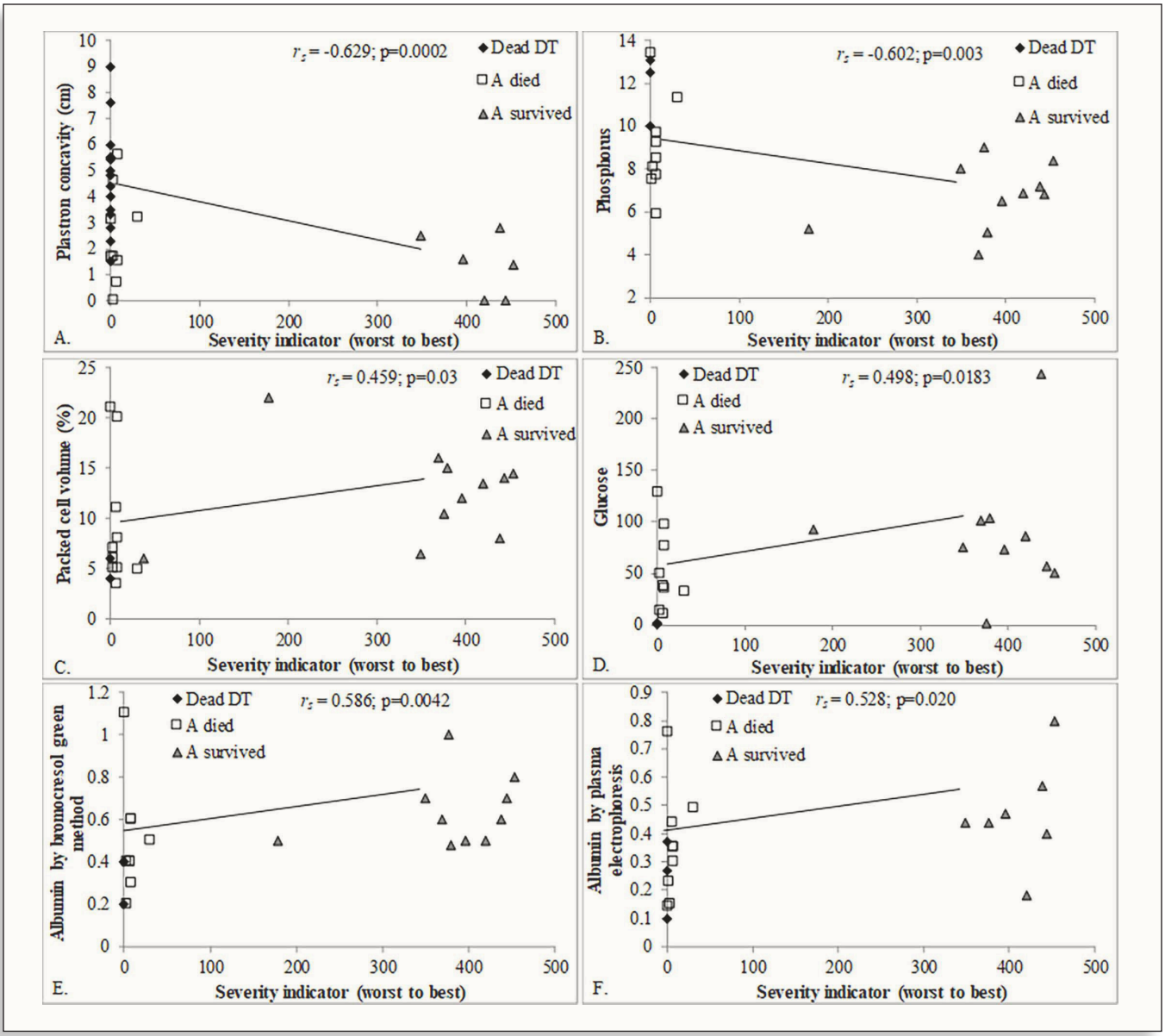

FIG 6. Health variables that significantly correlated with the severity indicator in debilitated loggerhead sea turtles (DTs). Spearman rank correlation coefficients and p-values are shown. (A) plastron concavity; (B) packed cell volume (PCV); (C) glucose; (D) phosphorus; (E) albumin by bromocresol green method; (F) albumin by plasma electrophoresis; (G) B-lymphocyte proliferation. https://doi.org/10.1371/journal.pone.0200355.g006

excretion of K. Possible explanations for the significantly lower K concentrations in other DTs include hyporexia or anorexia, and/or loss of K through kidneys and/or gastrointestinal tract.

Na and Cl were higher in dead DTs and lower at time point B compared to HTs (S3 Table). Likewise, UA was higher in dead DTs and A-died than HTs. This indicates that dehydration and/or renal insufficiency was present in the two most severe categories of DTs.

Elevated concentrations of certain enzymes in the plasma can indicate tissue damage. Plasma AST was higher in A-died and the B time point compared to HTs, and it was higher at admission than recovery (S3 Table). CK was significantly higher in A-died than recovered DTs or HTs. Both enzymes are widely distributed in tissues of loggerhead sea turtles, with highest concentrations of CK in heart, skeletal muscle, gastrointestinal tract and central nervous system, and highest concentrations of AST in heart, liver and kidney.[38] Concurrent elevations of both enzymes may indicate muscle injury/wasting or marked gastrointestinal disease.[38] Both of these organ systems are affected in debilitated turtles.

The low detection frequency of bile acids and lack of significant changes among DT categories (S3 Table) raises concern that the radioimmunoassay does not detect reptilian bile acids or alcohols and that a species-specific assay is needed. This is consistent with a recent study using the same assay in loggerhead turtles,[39] and highlights the need for a reptile-specific test method.

During biliverdin measurements, the limit of detection (LOD) changed between the time when the DT samples (LOD = 0.6 mg/dL) and the HT samples (LOD = 1.25 mg/dL) were analyzed. This

resulted in no detection of biliverdin in the HTs and challenges to interpret changes through rehabilitation. A-survived DTs had the highest biliverdin concentrations, and these were significantly higher than HTs (S3 Table). Of the three turtles admitted with green plasma, biliverdin measurements were available for two samples and these had the two highest concentrations, 4.7 and 9.3 mg/dl, indicating an association of biliverdin concentration and plasma discoloration. Reptiles lack biliverdin reductase and excrete biliverdin rather than bilirubin as in mammals, and thus this analyte has potential as a diagnostic indicator of compromised liver function. The assay, though, requires further optimization and validation.

Immune functions

Measuring immune functions is relatively new to the field of sea turtle biology, and few studies have performed hematology along with multiple immune functions that span both the innate and acquired immunity.[21,40] Data for lymphocyte proliferation (LP), a measure of acquired immunity, as well as respiratory burst and lysozyme activity, both measures of innate immunity, in the different turtle categories are shown in S4 Table. Respiratory burst activity is an indicator of innate immunity, infection, and inflammation. When assessed using NBT as described herein, it determines the production of superoxide formation from neutrophils, the cellular equivalents of reptilian heterophils, and monocytes.[41] Superoxide formation and release is critical in host defense to bacterial and parasitic infections and is increased in animals following antimicrobial challenges.[42] Plasma lysozyme activity is a marker of pro-inflammatory/inflammatory responses and a measure of innate immunity.[43,44] Lysozyme is produced by fish and mammalian neutrophils in response to bacteria.[43,44] Reptilian lysozyme can lyse and kill both gram-positive and negative bacteria.[45] Proliferation of T-and B-cells as measured by mitogen stimulation determines the ability of the adaptive immune system to mount a response as proliferation of these cells is a critical step in the process.

Both LPS-and PDB-induced LP were significantly suppressed in one or more of the DT categories at time of admission compared to HTs, while concurrently elevated respiratory burst and plasma lysozyme activity were also observed (S4 Table; Fig. 5). Specifically, respiratory burst (super oxide production) was significantly elevated at admission but rapidly declined, such that no difference from HTs was observed by time point B (Fig. 5C, S4 Table). Plasma lysozyme activity was significantly increased in DT compared to HTs throughout rehabilitation (Fig. 5D, S4 Table) supporting the suggestion of ongoing inflammation, as observed by the described leukogram changes (i.e., left-shifting). Elevated innate immunity has also been demonstrated in dehydrated Gila monsters (*Heloderma suspectum*) and those undergoing fat store loss.[46] It was suggested that the reptiles were actively elevating physiologically cheaper innate immune response strategies during stressful periods. It is possible DTs are using similar immune response mechanisms.

Through rehabilitation, LPS-and PDB-induced LP did not significantly change between A and D sample time points, but was significantly decreased in A-died samples compared to HTs (S4 Table). This suggests suppression of humoral immunity to both T-dependent and T-independent antigens in these turtles at stranding. Increased innate immunity functions could be an attempt to compensate for this deficit and/or be linked to inflammation. Interestingly though, LP stimulated with ConA (T-lymphocyte proliferation) for 5 days exhibited an increase during rehabilitation to levels that were an order of magnitude higher than HTs at time points C and D (Fig. 5A). The occurrence of compensation between branches of the immune system and the ability of the system to rebound after suppression from drugs, stress hormones, environmental stressors and malnutrition (fasting, starving, decreased protein intake) is well known.[47-52] Once the stressor is removed, the system will overcompensate to levels much higher than normal before returning to normal levels. Therefore, the observed increase in ConA-induced LP is most likely due to the rebound effect following the stressors associated with a prolonged disease state, malnutrition, and inflammation.

PHA-induced LP showed a similar pattern to ConA-induced LP through rehabilitation, but was not statistically different from HTs due to the generally higher standard deviation observed with PHA (Fig. 5B, S4 Table). Additionally, plasma lysozyme activity and numbers of monocytes all exhibited increases from A-survived to C followed by a slight drop at D; thereby, mirroring ConA- and PHA-induced proliferation patterns. The leukogram and immune function results complement each other. At stranding, the innate immune functions (respiratory burst and lysozyme) of DTs suggest activation, which is consistent with changes in indicators of inflammation, including increased heterophil toxicity and left-shifting (S2 Table), all of which stabilized and/or improved consequently during rehabilitation. The suppression of the adaptive immune system (decreased LPS-and PDB-induced LP at admission) matches the lower total lymphocytes (S2 Table) and gamma-globulins (S3 Table) seen at stranding in the DTs.

Overall, the data suggest that at stranding the innate immune system of DTs is activated (increased respiratory burst and lysozyme at admission) likely due to an immediate need to fight increased presence of parasites and bacteria and/or to compensate for suppressed humoral immunity. B-cell function, as indicated by LPS-and PDB-induced LP, was reduced in DTs that died compared to HTs, but no significant difference was observed between A-survived and HTs. These comparisons indicate that the turtles with the worst prognosis have the most severe B-cell suppression. Lymphoid depletion and reduced antibody production have been associated with starvation in chicks.[53] A similar mechanism can be presumed in chronically debilitated turtles. Suppression of any part of the immune system can increase susceptibility to infections, specifically to parasites when lymphocyte defects are present.[54] However, T-cell functions seemed to rebound following admission. These findings provide new information on the immune responses of sea turtles and more generally in reptiles, all

of which are understudied.[45] In particular the elevated and long-lasting increases in plasma lysozyme activity in DTs supports the idea that innate immunity antimicrobial proteins and associated mechanisms are potent and important defense mechanisms for reptiles.[25,45]

Prognostic indicators

The severity indicator calculated for DT dead, A-died, and A-survived provided a continuous variable to examine correlations with other health indicators. Those that are significantly correlated (S5 Table) with the severity indicator are considered to be the most prognostic. Fig. 6 visually shows the relationship for selected variables. Note that the most severe cases had the lowest severity indicator score (Fig. 1). Analytes with a significant correlation to worsening severity included greater plastron concavity (Fig. 6A), P (Fig. 6B), UA, and K, and reduced PCV (Fig. 6C), total WBC, Glc (Fig. 6D), TS, TP by Antech, ALB by bromocresol green method (Antech, Fig. 6E) and by protein electrophoresis (U. Miami, Fig. 6F), lymphocyte proliferation at 4 days (PDB 25 and 50) and at 5 days (PDB 25), alpha 1-globulins, beta-globulins, Ca:P, and biliverdin. Based on statistical analyses and clinical judgement, plastron concavity ≤ 2 cm, PCV ≥ 10%, P ≤ 9 mg/dL, ALB ≥ 0.4 g/dL, UA ≤ 2.4 g/dL, Glc ≥ 55 mg/dL, and TS ≥ 2 g/dL are the most predictive of a DT surviving rehabilitation. Unfortunately, measuring plastron concavity on live DTs is not recommended because of the high risk of cardiac tears from plastron bones that are more freely moveable from reduced connective tissue in DT.

Conclusions and clinical relevance

This study defined characteristic morphometrical and clinicopathological abnormalities of DTs at admission and through rehabilitation and identified clinically useful prognostic indicators. This new information is unique in that it reports a comprehensive database focused solely on DTs and sets itself apart from previous studies that have examined groups of loggerhead turtles that stranded from various causes.[8,11,26]

The clinical diagnosis of chronically debilitated loggerhead turtles is made simply by visual assessment of emaciation, lethargy, and heavy barnacle coverage on skin. At time of admission, severe metabolic derangements are observed and reflected in hematological and plasma chemistry profiles. Live patients have a guarded prognosis, with a documented mortality of 52% in this study, which highlights the challenges for treatment and rehabilitation. Plastron concavity and PCV are variables with substantial prognostic significance, but we encourage development of innovative methods for taking plastron measurements on live DTs while they are in normal upright position rather than turning them to dorsal recumbency and risking severe injuries.

Improvements in treatment strategies can hopefully be made based on our descriptions of the severe systemic pathophysiological effects in DTs. The animals require long-term care with the goals to reverse the emaciated condition and treat parasitic and bacterial infections. Treatment of the individual patient is based on the veterinarian's discretion, and the current consensus on best treatment strategies among experts include the use of a combination of crystalline and colloidal fluid therapy, total parenteral nutrition, and antimicrobial therapy early on in the course of rehabilitation.[10] Blood transfusions are reserved for severe cases (PCV ≤ 5%). Gradual introduction to de-beaked and de-boned seafood should be instituted. Once the turtle is eating regularly gastrointestinal protectants and oral antibiotics (metronidazole) are often used. Deworming agents such as fenbendazole should be utilized but not until the anemia has significantly improved and a regenerative response is noted.[10]

These recommendations for intense medical treatment from the literature and the findings of this study demonstrate the severe clinical condition of chronic debilitation, representing the end stage of starvation. Future studies are needed to further understand the initial cause(s), temporal and spatial trends, and the effects on the loggerhead sea turtle population in the Southeastern United States.

Supporting information

S1 Fig. Photos of a debilitated loggerhead sea turtle. Photographs of a dead debilitated loggerhead sea turtle (*Caretta caretta*) with (A) emaciation, sunken eyes, epibiota on head and skin, and (B) severely sunken plastron, poor body condition, and generalized epibiota growth on plastron and skin. (TIF)

S2 Fig. Scatterplots of different measures of plasma proteins in debilitated loggerhead sea turtles (DTs). Linear trendlines and equations are shown for each comparison along with the Spearman correlation coefficient and p-value. (TIF)

S3 Fig. Differences between different measures of plasma proteins in debilitated sea turtles (DTs). Mean and standard deviation of the difference between paired plasma samples from debilitated loggerhead turtles (DTs) for different measures of plasma proteins. PE = plasma electrophoresis; BG = bromocresol green. An asterisk indicates a significant difference between the two methods compared within each bar (Wilcoxon signed rank test, $p<0.05$). (TIF)

S1 Table. Morphometric data in debilitated sea turtles (DTs) stranded along the southeast U.S. compared to healthy control turtles. (DOC)

S2 Table. Hematological data in debilitated sea turtles (DTs) stranded along the southeast U.S. compared to healthy control turtles. (DOC)

S3 Table. Plasma chemistry data in debilitated sea turtles (DTs) stranded along the southeast U.S. compared to healthy control turtles. (DOC)

S4 Table. Immune function measurements in debilitated sea turtles (DTs) stranded along the southeast U.S. compared to healthy control turtles. (DOC)

S5 Table. Prognostic indicators determined by correlations with severity indicator. Spearman correlations between the severity indicator in debilitated loggerhead turtles (DTs) and hematology, plasma chemistry and immune function variables. Values

for the severity indicator decrease with worsening severity of debilitation. P-values in bold with * are significant. (DOC)

S1 File. Protocol for assessing debilitated loggerhead sea turtles. Written protocol, data-sheets, flowcharts. (PDF)

Acknowledgments

The authors thank the following individuals for their support and contributions to the study: Allen Foley, Sally Murphy, and Mark Dodd (workshop participants and reviewers), M. Bauer, J. Beasley, W. Cluse, T. Murphy, T. Sheridan, B. Stacy, C. Manire, S. Terrell, A. Tuttle, and C. Hope (sample collection), M. Bresette, W. Teas, and E. Jacobson (workshop participants), DuBose Griffin (sample collection and reviewer), C. Gregory and B. Ritchie (biliverdin analysis), M. Koperski (permits), B. Schroeder (funding), and P. Thompson (blood film staining), as well as technicians, volunteers, veterinarians and support personnel at rehabilitation facilities and stranding groups.

We thank the following organizations: the Sea Turtle Stranding and Salvage Network, SC Department of Natural Resources, NC Wildlife Resources Commission, Florida Fish and Wildlife Conservation Commission, Inwater Research Group, the Georgia Department of Natural Resources, and all the rehabilitation centers listed in the Methods section. We are grateful for the St. Catherines Island Foundation and the Wildlife Conservation Society for hosting the workshop in 2003.

Funding was provided by the U.S. National Oceanic and Atmospheric Administration's National Marine Fisheries Service. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclaimer: Certain commercial equipment, instruments, or materials are identified in this article to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Author Contributions

Conceptualization: Jennifer M. Lynch, Rusty D. Day, Craig A. Harms, Margie M. Peden-Adams, Al L. Segars, Terry M. Norton.

Data curation: Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, A. Michelle Lee, Margie M. Peden-Adams.

Formal analysis: Nicole I. Stacy, Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, Craig A. Harms, A. Michelle Lee, Margie M. Peden-Adams.

Funding acquisition: Terry M. Norton.

Investigation: Nicole I. Stacy, Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, Carolyn Cray, Rusty D. Day, Craig A. Harms, A. Michelle Lee, Margie M. Peden-Adams, Kelly Thorvalson, Al L. Segars, Terry M. Norton.

Methodology: Nicole I. Stacy, Jennifer M. Lynch, Carolyn Cray, Rusty D. Day, Craig A. Harms, A. Michelle Lee, Margie M. Peden-Adams, Kelly Thorvalson, Al L. Segars, Terry M. Norton.

Project administration: Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Craig A. Harms, Margie M. Peden-Adams, Kelly Thorvalson, Al L. Segars, Terry M. Norton.

Resources: Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, Craig A. Harms, Margie M. Peden-Adams, Kelly Thorvalson, Al L. Segars, Terry M. Norton.

Software: Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, Margie M. Peden-Adams.

Supervision: Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Craig A. Harms, Margie M. Peden-Adams, Kelly Thorvalson, Al L. Segars, Terry M. Norton.

Validation: Nicole I. Stacy, Jennifer M. Lynch, Craig A. Harms, Margie M. Peden-Adams, Terry M. Norton.

Visualization: Jennifer M. Lynch, Margie M. Peden-Adams, Terry M. Norton.

Writing (original draft): Nicole I. Stacy, Jennifer M. Lynch, Terry M. Norton.

Writing (review & editing): Nicole I. Stacy, Jennifer M. Lynch, Michael D. Arendt, Larisa Avens, Joanne Braun McNeill, Carolyn Cray, Rusty D. Day, Craig A. Harms, Margie M. Peden-Adams, Al L. Segars, Terry M. Norton.

Funding

Funding was provided by the U.S. National Oceanic and Atmospheric Administration's National Marine Fisheries Service. 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.

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Author Affiliations

[1]Dept of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA.

[2]National Institute of Standards and Technology, Chemical Sciences Division, Hollings Marine Laboratory, Charleston, SC, USA.

[3]South Carolina Department of Natural Resources, Charleston, SC, USA.

[4]NOAA, National Marine Fisheries Service, Beaufort Laboratory, Beaufort, NC, USA.

[5]Division of Comparative Pathology, Department of Pathology & Laboratory Medicine, University of Miami School of Medicine, Miami, FL, USA.

[6]North Carolina State University, Center for Marine Science and Technology, Morehead City, NC, USA.

[7]Medical University of South Carolina, Charleston, SC, USA

[8]South Carolina Aquarium, Charleston, SC, USA.

[9]Georgia Sea Turtle Center, Jekyll Island Authority, GA, USA.

[10]St. Catherines Island Foundation, Midway, GA, USA.

[a]Currently: Trident Technical College, Charleston, SC, USA.

[b]Currently: Peden-Adams and Assoc., LLC, Charleston, SC, USA

[c]Currently: NIST, Chemical Sciences Division, Hawaii Pacific University, Kaneohe, HI, USA.)

Wildlife rehabilitation: A case study of the neotropical opossum, *Didelphis marsupialis insularis,* Allen 1902

L. Tardieu[a]*, W. Rollock[b], G. W. Garcia[a]

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[a]Open Tropical Forage-Animal Production Laboratory (OTF-APL), Department of Food Production (DFP), Faculty of Food and Agriculture (FFA), University of the West Indies, St. Augustine, Trinidad and Tobago.
[b]The Wake up and Call Shelter, Stratmore Avenue, Glencoe, Trinidad and Tobago.

ABSTRACT: Wildlife rehabilitation facilities in the Caribbean region are limited, yet they can provide relevant information on wild populations. *Didelphis marsupialis insularis* is a popularly hunted, under-studied, neotropical marsupial species that is increasingly being admitted for rehabilitation. The aim of this study was 1. To record the experiences of rehabilitating *D. marsupialis insularis* in the neotropical island of Trinidad and Tobago and 2. To extract and highlight information on the biology of this opossum subspecies. Using admission records, obtained over a roughly four-year period, two breeding periods (February to March and August to October) were illustrated. Litter sizes averaged five individuals, with a range of one to eight young. This species was found to be common in urban areas of the country, with dog attacks reported as the major cause for admission. Thus the information recorded by this wildlife rehabilitation facility has provided great insight on the sparsely studied opossum, *D. marsupialis insularis.*

KEYWORDS: *Didelphis marsupialis insularis*; neotropical wildlife; opossum; wildlife rehabilitation

CORRESPONDING AUTHOR:
Laura Tardieu
OTF-APL
Department of Food Production
Faculty of Food and Agriculture
University of the West Indies
St. Augustine, Trinidad and Tobago
Email: l_tardieu@hotmail.com

Reprint (open access): Tardieu L, Rollock W, Garcia, GW. *Brazilian Journal of Biology.* Epub September 26, 2019. https://doi.org/10.1590/1519-6984.214757.

Introduction

Wildlife rehabilitation

Wildlife rehabilitation can be defined as "the temporary care of injured, diseased and displaced indigenous animals and the subsequent release of healthy animals to an appropriate habitat in the wild".[1,2] It can vary in scope from individual rehabilitators, to large, well-equipped and well-staffed, modern animal hospitals.[3,4]

Worldwide, wildlife rehabilitation is viewed as a useful conservation tool for protec-

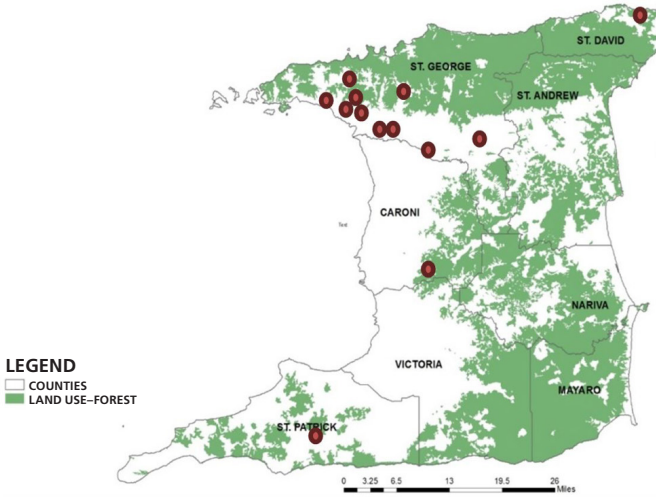

FIGURE 1. Map of Trinidad showing locations of opossums admitted into the Wake Up and Call Shelter (WUC).

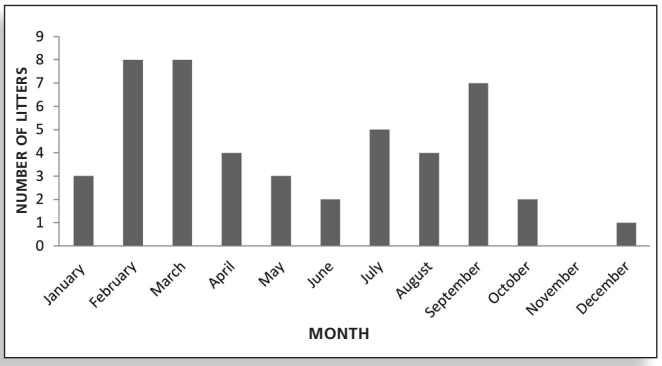

FIGURE 2. Estimated birth month based on young opossums admitted to the WUC for the period 2014–2017.

tion of "wild" species.[5] However, wildlife rehabilitators can play a number of other important roles. They can provide knowledge on the biology and behavior of wildlife species and educate the public on the value of wildlife and the roles they play in healthy ecosystems.[6]

Additionally, they can potentially detect infectious diseases, identify ecological changes and anthropogenic effects, and thus act as monitors for wildlife diseases and ecosystem health.[4,7] As studies have shown, even the single description of a disease, reported by a wildlife rehabilitator, can be of significant importance by being an early indicator of a disease outbreak in the area.[4,8]

While there are numerous wildlife rehabilitation facilities in developed nations, developing nations, like those in the neotropics, have limited wildlife rehabilitation resources.[9] Yet increased hunting and overexploitation of local wildlife have threatened, or placed under threat, wildlife populations in these regions.[10] As highlighted above wildlife rehabilitators can provide one form of relief for animals, but more importantly, they can provide vital biological information on understudied species and further serve to monitor the population health of widely hunted species.

In the Caribbean islands of Trinidad and Tobago, there are few recognized wildlife rehabilitation centers that focus mainly on indigenous wildlife species. Records from these centers are an unexploited resource that can provide crucial information on the health and status of wildlife populations. This is particularly relevant given the sparse information available on the biology of many Caribbean species. Scientific documentation in the form of case studies are therefore a significant first step in recognizing and utilizing the vital information stored in the notes and accounts of wildlife carers.

Didelphis marsupialis insularis

Locally known as the "manicou," *D. marsupialis insularis* is found in the Caribbean region. A common inhabitant of Trinidad and Tobago, this marsupial can be seen in both rural and urban areas.[11,12,13] Regarded as a pest species, the "manicou" is listed as "Vermin" under the Conservation of Wildlife Act[14] in the Laws of Trinidad and Tobago, and can therefore, by law, be killed year-round on private lands on both islands. As a valuable source of protein for locals, it is a widely sought-after and hunted species; and yet very little information has been documented on this native opossum.[15,16] The end result is a dearth of knowledge on this species in the Caribbean.

The objectives of this paper were to document one wildlife center's experience with rehabilitating the neotropical *D. marsupialis insularis* and to highlight important biological information on this species, including the description of a parturition that occurred in captivity.

Materials and methods

A retrospective study was conducted using data obtained from one rehabilitation shelter, the Wake Up and Call Shelter (WUC) located in Trinidad and Tobago (10.6918° N, 61.2225° W). The WUC is a small facility (<40 animals a year) that focuses on caring for local wildlife species and particularly specializes in opossum (*Didelphis* spp.) care. Data were collected from May 2014 to October 2017. The following variables were considered: age at admission, location the specimens were found, date of admission, sex, litter size, cause of admission, and outcome after treatment. Causes of admission were identified based on those reported at admission and on physical examination. Animal management records on captive-held adults were also examined for behavioral information. Descriptive statistics and inferential analyses were used and means ±SD are provided throughout the manuscript when necessary.

Results

Over the study period, a total of 150 young opossums were admitted for rehabilitation to the WUC, with an average of 37.5 per year. The majority of opossums admitted to the WUC were received from the Northwestern region of Trinidad (81%, n=98) (Fig. 1). The year 2015 held the highest number of admissions (30%, n=45), and over the study period the most admitted opossums were newborns less than one week old (20%, n=30). Litters were usually brought in without their mothers, and averaged (4.69±2.5, n=32) in size with a range of one to eight young. Based on the

ages of the young admitted, it was possible to extrapolate the birth months, and two breeding periods could be identified: February to March, and August to October (Fig. 2).

A slightly greater number of young males (2.45±1.36, n=76) were admitted, over the total study period, than females (2.06±1.75, n=64). The cause of admission was found to be mainly dog attacks (34%, n=51), followed by car accidents (18%, n=27) and hunting (17%, n=26) (Table 1).

The three outcomes after admission into the WUC were death, release, and kept in captivity. The majority of young opossums that were admitted for care died due to unknown causes (35%, n=52); closely followed by physical damage, probably inflicted while the young were being removed (by human carers) from the mother opossum's nipples (31%, n=46) (Table 2). Only a small portion of the total young admitted survived (29%, n=44) and were released, over the four-year period (21%, n=32) (Table 2).

Parturition in captivity

In early July 2016, one breeding episode occurred between two adult, captive opossums. The breeding pair consisted of a female (two–three years old) and a male (one year old). Both adults were wild born, but had been brought into WUC as young and retained at the clinic. The WUC at that time held four adult males and one mature female in their care.

Indications that the female was in estrus were first observed when the four males produced a series of vocalizations in the form of "metallic" clicking noises in late June 2016. The female was also noted to have ceased eating around this time. To determine the interest of the female in breeding, the four males were individually introduced to the female in the last week of June 2016. This was done via a "limited contact" method devised by the WUC, described as follows: males, penned individually in smaller cages, were sequentially placed inside the larger female cage. This served to protect the males from the highly aggressive behavior of the female, since females only tolerate males during periods of receptivity.

The female indicated, or chose by lack of aggressive behavior, the male that she was interested in. This male was then allowed access, via the opening of his cage, to the female's enclosure on the 5th July 2016. The male, on entering the female's enclosure, proceeded to mark the enclosure. Mating occurred on the first night the male was introduced, and the male was removed the next morning (6th July 2016) from the female's enclosure.

Eleven days later (16th July 2016) at 0830 h (GMT-4), signs of parturition were observed, with the female's stomach displaying abdominal contractions (spasms and movement by the pouch area) in a similar pattern as described by Reynolds.[17] The female was observed on many instances subsequent to this observation licking her pouch area.

In early September 2016, one newborn's tail was observed in the female's pouch. Subsequent observation however, found no more evidence of young in her pouch and it was concluded that the young had died.

TABLE 1. Causes of admission of *D. marsupialis insularis* to the WUC over the period 2014-2017.

CAUSE OF ADMISSION	N	%
Car accident	27	18
Dog attack	51	34
Hunting	26	17
Injured	3	2
Unknown	41	27
Mother abandoned	2	1
TOTAL	**150**	**100**

TABLE 2. Outcome of *D. marsupialis insularis* admissions into the WUC over the period 2014–2017.

OUTCOME	N	%
Cannibalism	2	1
Damaged spine	1	1
Dead on arrival	5	3
Physical damage/removal from mothers' nipples	46	31
Unknown	**52**	**35**
Total Deaths	**106**	**71**
Released	**32**	**21**
Kept in captivity/Still being treated	**12**	**8**
Total Admitted	**150**	**100**

Discussion

Much of the records supplied by the WUC have provided new reproductive and behavioral information on this species. This included breeding periods which were extrapolated using the ages of the young opossums admitted for rehabilitation. Based on this data, at least two defined breeding seasons, February to March and August to October, were identified for *D. marsupialis insularis,* and both the mean litter size and range were found to be fairly similar to other South American *Didelphis* spp.[18,19]

Over the study period, the causes of admission were anthropogenic activities, and varied from hunting to car accidents to dog attacks. This is supported by recent research, which also identifies anthropogenic factors as one of the most predominant causes of wildlife admissions worldwide.[20,21] Dog attacks were the highest cause of admission at the WUC, and may be due to opossums living in residential areas where dogs are commonly found. This is supported by the admission records that list urban areas of Trinidad and Tobago as the primary origins of the admitted individuals.

Similarly, congeners of the local opossum, namely *D. albiventris, D. pernigra,* and *D. virginiana,* have all been noted as being very tolerant of human-occupied areas. They have commonly been identified near human dwellings, garbage dumps, and agricultural lands.[22,23] In contrast, studies show that *D. marsupialis* appears

to avoid human-occupied areas.[24,25,26,27]

"Unknown" causes were one of the main reasons for opossum mortality after admission. One possible explanation could have been illness or diseases that arose due to separation from the mother. Studies have found that newborns are able to obtain passive immunity from viral diseases, like vesicular stomatitis, through their mother's milk.[28] As the majority of newborns were admitted after the death of their mothers, they may not have been able to obtain the antibodies from their mother's milk and consequently may have had increased susceptibility to diseases.

Another main source of mortality after admission was physical/internal damage caused by human carers while removing young from the female's nipples. This is not unanticipated, as newborns are very vulnerable during the "fixation period." At this stage the newborn's mouth exhibits partial lip fusion[29,30] and the lips fundamentally tear once the young are removed from the nipples. In addition, their bodies are very fragile, so that even with careful handling damage cannot be totally avoided. One suggestion to reduce this cause of mortality would be to remove the nipple from the female with the young still attached, and using a small tube, feed the young through the nipple. However, this technique requires testing to determine its feasibility. As a result of high mortality after admission, the success rate of releases was considered low.

The one instance of parturition that occurred at the WUC proved both insightful and informative. Although unsuccessful, as far as the authors are aware, this is the first documented instance of reproductive behavior and activity of *D. marsupialis insularis* in captivity. Much of the observations of the WUC during this parturition episode, including the intolerance and aggressiveness of the female against the males, the emission of sexual vocalization, cage-marking behavior by males, and the post-birth licking behavior of the female, were similar to previous descriptions on pre- and post-pregnant *Didelphis* spp.[17,29,31,32,33,34,35] However, the gestation period observed in this study was notably shorter (11 days), as compared with the previously stated gestation period of 12–15 days in captive held *D. virginiana* and *D. marsupialis*.[26,29,36,37]

The clicking vocalizations displayed by males during the females estrous were a sign of mating behavior that was not previously recorded for this species. It has, however, been observed in the male North American opossum in association with sexual interaction.[17,33,38,39] The change of eating habits observed in the female was also not previously stated in the literature, and combined with the vocalizations emitted by males, could be used to indicate the readiness of the *D. marsupialis insularis* female for mating under captive settings.

It can be inferred that the death of the only newborn that had reached the female's pouch was due to starvation. This is supported by Reynolds[17] who found that it took two or more suckling young to sustain lactation in the female opossum. The small litter size produced in this case may have been a result of the advanced age of the female, which has been shown to affect fertility in North American opossums.[17]

In conclusion, the data gathered at the facilities of the WUC have provided interesting biological insight into some aspects of the under-studied and increasingly hunted Caribbean "manicou." This information, combined with further population studies, can be used to develop effective conservation management plans for this species in the future.

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In the News

CONTINUED FROM PAGE 6

and Furrows, their contact information and wildlife coverage remain the same.

Effects of Deepwater Horizon Remain

NEW ORLEANS, USA (April 7, 2020)—A new National Wildlife Federation report, "10 Species, 10 Years Later: A Look at Gulf Restoration after the Deepwater Horizon Disaster," summarizes the latest information available about ten wildlife species that were affected by the spill as well as the restoration efforts underway.

"For many wildlife in the Gulf, the decade-old Deepwater Horizon oil spill is not over. We will probably never understand the full extent of the damage, but we do know that we have an obligation to restore the Gulf of Mexico and to ensure that a disaster on this scale never happens again," said David Muth, director of the National Wildlife Federation's Gulf of Mexico Restoration Program.

The report describes several species that are still struggling a decade after the disaster:

■ The endangered Kemp's ridley sea turtle's once-promising recovery seems to have halted in 2010. Before 2009, Kemp's ridley nests were once increasing at a rate of 19 percent a year on average; nesting has been erratic since the spill.

■ Coastal bottlenose dolphins in oiled areas are still sick and dying a decade later. Successful births remain less than a quarter of normal levels.

■ Corals in several locations—including some colonies that are more than six centuries old—still show signs of oil damage and are not expected to recover.

■ An estimated 17 percent of the Gulf's tiny population of Bryde's whales died as a result of the oil spill, and scientists predict reproductive failures among exposed whales that survived. The population was listed under the Endangered Species Act after the disaster.

"It's important to continue to study the impacts of the spill so we can understand how to better protect the Gulf in the future," said Jessica Bibza, policy specialist on the National Wildlife Federation's Gulf of Mexico restoration program. "Many questions about the impacts of the oil spill on wildlife and habitats remain unanswered to this day."

Call for Koalas to be Listed as Endangered

BARNSTABLE, Massachusetts, USA (April 4, 2020)—The World Wide Fund for Nature–Australia (WWF-Australia), the International Fund for Animal Welfare (IFAW), and Humane Society International (HSI), have provided strong evidence to the federal government to support the nomination.

This includes a new report estimating Queensland's koala population has crashed by at least 50% since 2001 because of deforestation, drought and the recent bushfires.

It also found:

■ Forest fires in the Sunshine State killed a minimum of 672 koalas between August and December 2019,

■ Koalas appear to be functionally extinct in central Queensland's Mitchell Grass Downs bioregion, and

■ It is estimated there has been an 80% decline across the Mulga Lands in the state's south-west, previously considered to

support the second highest proportion of koalas across all bioregions in Queensland.

A parallel analysis released by IFAW in early March, and just updated through 13 February, found the New South Wales koala population has also suffered a decline of between 33% and 61% since 2001, with a conservative estimate of 6382 koalas killed in the 2019–20 bushfire season.

Koala populations in Queensland, New South Wales and the Australian Capital Territory were listed Vulnerable under the federal government's EPBC Act in May 2012. Since then, koalas have suffered relentless ongoing pressure. Land clearing has ramped up, increasing 13-fold in New South Wales since the government weakened native vegetation laws in 2016.

Climate change has supercharged droughts and heat waves, increasing koala deaths as their feed trees die off and waterways where koalas drink dry out.

Koalas were already on the path to extinction in eastern Australia. Then came the 2019–2020 bushfires when the koala became the unfortunate wildlife icon of the crisis both internationally and domestically. Many important populations were directly in the path of the fires, and may not recover without serious and long term rescue efforts.

It is understood the federal government's Threatened Species Scientific Committee is already considering the conservation status of the koala.

Three Million Online Listings for Trafficked Wildlife Removed by Tech/NGO Coalition

WASHINGTON, DC (March 2, 2020)—"Offline and in the Wild," a report just released about progress made by companies involved in the World Wildlife Fund (WWF), TRAFFIC, and International Fund for Animal Welfare (IFAW)-convened coalition, finds that efforts taken by these companies are helping to shut down the cloud-based trade routes cybercriminals rely on for exploiting wildlife.

The Coalition's progress has resulted from strengthened wildlife policies, an increase in staff ability to detect potential illegal wildlife products and live wild animals, regular monitoring and data sharing from wildlife experts, reports sent in by volunteers through the Coalition's Wildlife Cyber Spotter Program, enhanced algorithms—thanks to key search word monitoring and collation—and shared learning.

"Criminal networks are taking advantage of internet platforms at the expense of the rarest species nature has to offer," said Crawford Allan, Senior Director for TRAFFIC at WWF. "But the vastness of the internet presents a challenge for law enforcement to regulate. The online companies in our Coalition now have the tools to fight back against wildlife trafficking online, and can help ease the burden on law enforcement."

The Coalition to End Wildlife Trafficking Online was born out of the global proliferation of internet access and resulting shift in illegal wildlife trade transactions from physical to online markets. The extensive number of listings removed by the Coalition's second anniversary demonstrates both the long-term effectiveness of the partnership and the continued commitment of the companies to prevent wildlife trafficking on their platforms.

According to Tania McCrea-Steele, International Project Manager, Wildlife Crime at IFAW,

> "Uniting online technology companies is critical in the fight against wildlife cybercrime as wildlife traffickers are abusing the anonymity of the internet to exploit endangered wildlife. Tragically, you can find elephant ivory, pangolin scales, live tiger cubs, live birds and reptiles and more, all for sale on your smart phone. The online technology companies are a core part of the solution as they are able to work at an unprecedented global scale and disrupt illegal wildlife trafficking."

The Coalition has also created a site for members of the public to report potential trafficked wildlife sales. Prohibited wildlife products found online can be flagged for removal at https://www.endwildlifetraffickingonline.org/. WWF, IFAW and TRAFFIC train citizen science volunteers on how to identify prohibited wildlife products online through the Coalition's Wildlife Cyber Spotter Program.

Sea Alarm Recounts Lessons Learned in Cooperation at 20

BRUSSELS, Belgium (March 30, 2020)—In its 20-year history Sea Alarm has been active across the globe, carrying out numerous activities to advance wildlife response preparedness with a large number of stakeholders.

Sea Alarm works with industry, NGOs and governments toward advancing the quality of oiled wildlife response planning and preparedness. These three stakeholder groups depend on each other when confronted with an incident—the incident binds them together. While Sea Alarm's target has always been to bring wildlife responders together to discuss experiences of good practice and explore grounds for cooperation and joint response capability, it was clear from the start that governments and industry should be on board to make things work.

Sea Alarm's method is one of dialogue, working with key actors to identify obstacles, and finding ways to unblock these obstacles. They write,

> "An important element in these conversations has been to share our experience, our knowledge and our insights, and subsequently to learn from our partners, and jointly find solutions for improvement. What we learned in one country could be shared with stakeholders in another country. Things that worked in that other country could be shared with the next. In this way our insights have grown over time, while our messages have been modified accordingly."

The NGO has learned how to distinguish between generic values/concepts on the one hand, and on the other, the variability in the ways by which these values or concepts are perceived, depending on the cultural setting. They learned that what is conceived as totally logical in one country or culture is not always understood similarly by another. Therefore, developing

SELECTED ABSTRACTS

Adaptive changes in the genomes of wild rabbits after 16 years of viral epidemics

N Schwensow, S Pederson, D Peacock, B Cooke, and P Cassey. *Mol Ecol.* 2020. Accepted Author Manuscript. https://doi:10.1111/mec.15498

Since its introduction to control overabundant invasive European rabbits *(Oryctolagus cuniculus)*, the highly virulent Rabbit Haemorrhagic Disease Virus (RHDV) has caused regular annual disease outbreaks in Australian rabbit populations. Although initially reducing rabbit abundance by 60%, continent-wide, experimental evidence has since indicated increased genetic resistance in wild rabbits that have experienced RHDV-driven selection. To identify genetic adaptations, which explain the increased resistance to this biocontrol virus, we investigated genome-wide SNP (single nucleotide polymorphism) allele frequency changes in a South Australian rabbit population that was sampled in 1996 (pre-RHD genomes) and after 16 years of RHDV outbreaks. We identified several SNPs with changed allele frequencies within or close to genes potentially important for increased RHD resistance. The identified genes are known to be involved in virus infections, immune reactions or had previously been identified as being differentially expressed in healthy vs. acutely RHDV-infected rabbits. Furthermore, we show in a simulation study that the allele/genotype frequency changes cannot be explained by drift alone, and that several candidate genes had also been identified as being associated with surviving RHD in a different Australian rabbit population. Our unique dataset allowed us to identify candidate genes for RHDV resistance that have evolved under natural conditions, and over a time span that would not have been feasible in an experimental setting. Moreover, it provides a rare example of host genetic adaptations to virus-driven selection in response to a suddenly emerging infectious disease.

No longer a leap in the dark: the importance of protein as an energy source in amphibians

A Brenes-Soto, ES Dierenfeld, A Muñoz-Saravia, GPJ Janssens. *Wildlife Biology,* 2019(1):1–9. https://doi.org/10.2981/wlb.00551

Amphibian nutrition has been highlighted as one of the disciplines requiring more investigation to support ex situ conservation programs. Specifically, anuran metabolism related to dietary nutrients is not yet investigated in detail. Thirty (n = 30) free-range frogs from four families (Telmatobiidae, Hylidae, Leptodactylidae, Bufonidae) were collected in Bolivia, and opportunistic blood samples were drawn to determine acylcarnitines and amino acid profiles in order to evaluate metabolic activity. The overall profiles showed Telmatobiidae with higher numerical values of amino acids, while comparison with Hylidae displayed differences ($p < 0.05$) in metabolites related to amino acid catabolism, suggesting specific ketogenic pathways in Telmatobiidae as an adaptation to its extreme environmental (hypoxic) conditions. Multivariate analysis demonstrated both lipids and amino acids as the main forces in frog energy metabolism, confirming the carnivorous nature of anurans. Pathways detecting free carnitine and long chain acylcarnitines driving fat metabolism, as well as protein-derived utilisation of amino acid catabolites documented glucose sparing and energy production through both proteinogenic and ketogenic routes. Moreover, malonyl carnitine is suggested to play a role as a modulator of food intake and feeding status of frogs.

Zoonotic disease exposure risk and rabies vaccination among wildlife professionals

S Tarrant, J Grewal, H Yaglom, et al. *EcoHealth.* 2020;17:74–83. https://doi.org/10.1007/s10393-020-01469-w

More than 70% of zoonotic diseases are wildlife-associated, putting wildlife professionals at increased risk of occupational exposure. In 2008 and 2018, the Arizona Department of Health Services surveyed Arizona wildlife professionals from multiple agencies to assess the risk of disease exposure, rabies pre-exposure prophylaxis (PrEP) history, personal protective equipment (PPE) use, and zoonoses knowledge. In 2008, a 12-question survey was distributed at a state wildlife professional meeting using an anonymous email link. In 2018, a 20-question survey was distributed using an anonymous email link to wildlife agency employees. We received 164 and 81 complete responses in the 2008 and 2018 surveys, respectively. Bites from rabies reservoir or spillover species were higher in 2008 (42%) than in 2018 (16%). More respondents received PrEP in 2018 (53%) than in 2008 (45%). Among 43 respondents who performed necropsies or collected animal samples within the past 5 years (2014–2018), only 60% always wore latex or nitrile gloves, and 79% never wore a facemask. Respondents indicated lower awareness of certain zoonoses, including brucellosis (72%) and leptospirosis (60%). Results on zoonoses awareness and reasons for non-use of PPE highlighted targets for education to improve practices, including facilitation of PPE training to prevent future disease transmission.

Ear mite infection is associated with altered microbial communities in genetically depauperate Santa Catalina Island foxes *(Urocyon littoralis catalinae)*.

AL DeCandia, LJ Brenner, JL King, and BM vonHoldt. *Molecular Ecology.* 2020;29(8):1463-1475; https://doi.org/10.1111/mec.15325

The host-associated microbiome is increasingly recognized as a critical player in health and immunity. Recent studies have shown

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preparedness for wildlife response cannot be simply laid out as a blueprint programme in the sense of "one size fits all." A Sea Alarm spokesperson notes,

> "You only can assist if you are interested in learning, and expertise is a very limited qualification. Having been in many places in the world, having worked with many parties in different countries, looking at a range of ecosystem and cultural settings, has made us humble about thinking there is only one single approach with universal value."

Sea Alarm sees their mission as maximizing the potential for NGOs, governments, and industry to find ways to cross their respective borders and work together. Please visit www-sea-alarm.org for further details. ■

that disruption of commensal microbial communities can contribute to disease pathogenesis and severity. Santa Catalina Island foxes *(Urocyon littoralis catalinae)* present a compelling system in which to examine microbial dynamics in wildlife due to their depauperate genomic structure and extremely high prevalence of ceruminous gland tumors. Although the precise cause is yet unknown, infection with ear mites (*Otodectes cynotis)* has been linked to chronic inflammation, which is associated with abnormal cell growth and tumor development. Given the paucity of genomic variation in these foxes, other dimensions of molecular diversity, such as commensal microbes, may be critical to host response and disease pathology. We characterized the host-associated microbiome across six body sites of Santa Catalina Island foxes, and performed differential abundance testing between healthy and mite-infected ear canals. We found that mite infection was significantly associated with reduced microbial diversity and evenness, with the opportunistic pathogen *Staphylococcus pseudintermedius* dominating the ear canal community. These results suggest that secondary bacterial infection may contribute to the sustained inflammation associated with tumor development. As the emergence of antibiotic resistant strains remains a concern of the medical, veterinary, and conservation communities, uncovering high relative abundance of *S. pseudintermedius* provides critical insight into the pathogenesis of this complex system. Through use of culture-independent sequencing techniques, this study contributes to the broader effort of applying a more inclusive understanding of molecular diversity to questions within wildlife disease ecology.
[Associated article from National Science Foundation: Microbes linked to cancer in threatened California foxes: Can staph microbes lead to cancer?]

Asian elephant rescue, rehabilitation and rewilding

L Baker and R Winkler. *Animal Sentience.* 2020; 28(1). https://animalstudiesrepository.org/animsent/vol5/iss28/1/

Thailand has fewer than 10,000 elephants left. More of them are living in captivity to serve the tourist industry under grim conditions than are living free in what is left of their wild habitat. Conservation efforts need to be focused on all surviving members of the species, captive and free, but they need to take into account the inextricable entanglement of human and nonhuman animal lives in Thailand today. There is an opportunity for rescuing, rehabilitating and reintroducing captive elephants to the wild with the help of the traditional expertise of a mahout culture that has been elephant-keeping for centuries. We advocate a state of wildness that is meaningful to the elephants and can be attained in a way in which both elephant and human cultures are valued. This would be far better than the status quo for the elephants, restoring to them a life worth living.

"Good" and "Bad" Urban Wildlife

G Perry, C Boal, R Verble, and M Wallace. "Good" and "Bad" Urban Wildlife. In: FM Angelici and L Rossi (eds), Problematic Wildlife II. Springer, Cham; 2020:141-170. https://doi.org/10.1007/978-3-030-42335-3_5

Urban environments offer habitat for many species of animals. Although some of those are ubiquitous and/or undesirable, others are native and in some cases, of conservation value. In many cases, urban wildlife populations are a source of enjoyment for human residents, who sometimes invest considerable amounts in attracting them to yards and public spaces. Their presence there can serve an important educational role that helps protect non-urban habitats and species. Nonetheless, urban wildlife must survive what has been termed a "landscape of fear." Although some of the urban wildlife that do well in this environment are benign, other populations—sometimes of a species that, in other locations, is iconic and desirable—can become problematic. Some species can serve as vectors that carry important zoonoses, such as the plague or diseases that affect other wildlife. Others can create noise or olfactory nuisances and degrade structures or usability of public spaces. Some pose hazards at busy airports, whereas still others may present an envenomation or predation risk on unwary humans. Here, we review the role that reptiles, birds, and mammals play in urban environments and discuss how urban wildlife rehabilitation centers help address some related issues. We close by looking ahead and trying to predict how global patterns such as increased urbanization and population growth may affect urban wildlife and its value for conservation.

Unmonitored releases of small animals? The importance of considering natural dispersal, health, and human habituation when releasing a territorial mammal threatened by wildlife trade

M Campera, E Brown, MA Imron, and KAI Nekaris. *Biological Conservation.* 2020;242:108404. https://doi.org/10.1016/j.biocon.2019.108404

Unmonitored release is a common practice, especially in small animals, that present a series of adverse conditions if not well-planned. Small research centers and non-governmental organizations in developing countries often receive animals that are then subject to unmonitored releases. We explored the patterns of post-release and natal dispersal in the Javan slow loris, a Critically Endangered venomous and territorial mammal that is highly threatened by wildlife trade. We then determined the importance of health status and human habituation for the survival of translocated and natally dispersing animals. We collected data from 2012 to 2018 on pre-release and pre-dispersal health conditions and human habituation, post-release and post-dispersal presence of wounds, behavior, and ranging patterns of 11 translocated and 11 natally dispersing individuals and compared them with 12 stable resident individuals. Translocated animals had a larger home range size (15.9 ± 4.1 ha) and higher wound presence during recaptures (0.47 ± 0.13) than stable resident individuals (3.2 ± 3.0 ha; 0.10 ± 0.06) but they did not differ from natally dispersing individuals (13.8 ± 3.7 ha; 0.28 ± 0.11). Both translocated and natally dispersing individuals can move to a different habitat type compared to their release area or natal range. The fate of both translocated and natally dispersing individuals was influenced by their health state ($p < 0.001$), and human habituation significantly affected the possibility of being captured for wildlife trade of translocated individuals ($p = 0.048$). We highlight the importance of considering natal dispersal, health state, and human habituation before the release of small animals to avoid death and capture for wildlife trade. ■

TAIL END

Wilbur knew his insect cache would be safe here.

Acorn Woodpecker (*Melanerpes formicivorus*)

INSTRUCTIONS FOR AUTHORS

POLICY Original manuscripts on a variety of wildlife rehabilitation topics (e.g., husbandry and veterinary medicine) are welcomed. Manuscripts that address related topics such as facility administration, public relations, law, and education are invited as well.

Associate editors and anonymous reviewers, appropriate to the subject matter, evaluate each submitted manuscript. Concurrent submission to other peer-reviewed journals will preclude publication in the *Journal of Wildlife Rehabilitation* (JWR). The International Wildlife Rehabilitation Council (IWRC) retains copyright on all original articles published in the JWR but, upon request, will grant permission to reprint articles with credit given to the IWRC–JWR.

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Include the name of each author. Specify the corresponding author and provide affiliation, complete mailing address, and email address. The affiliation for all authors should be included in a brief (maximum of 100 words) biography for each that reflects professional experience related to rehabilitation or to the manuscript subject matter rather than personal information. Biographies may be edited due to space limitations.

Include an abstract that does not exceed 175 words and choose several (up to 14) key words.

Templates have been developed for the following submission categories: case study, technique (including diets), research, and literature review; authors may request a copy of one, or all, of these templates from the editor (jwr.editor@theiwrc.org) before developing a manuscript for submission to the JWR.

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