Supplement

Detailed Methodology

Lysozyme analysis

Lysozyme activity in plasma samples was measured using modifications of standard turbidity assays performed by Keller et al. (2006). A 1 mg ml^{-1} stock solution of hen egg white lysozyme (HEL; Sigma-Aldrich, St. Louis, Missouri USA) was prepared fresh in 0.1 M phosphate buffer (pH 5.9). *Micrococcus lysodeikticus* (Sigma-Aldrich) cell suspension was prepared by dissolving 50 mg of lyophilized cells in 100 ml of 0.1 M phosphate buffer. Hen egg white lysozyme was serially diluted by successive 1:1 dilutions in phosphate buffer to produce a standard curve of 0, 0.31, 0.63, 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 mg ml^{-1}. Aliquots (25 µl) of each concentration and 25 µl of test plasma were added to a 96-well plate in quadruplicate. *M. lysodeikticus* solution (175 µl) was quickly added to the first three rows of the sample wells and to each of the standard wells. The same amount of phosphate buffer was added to the fourth sample well to serve as a blank. Absorbance was measured at 450 nm using a BioTek® Synergy™ H1M microplate reader (BioTek US, Winooski, Vermont USA). Readings were conducted immediately (T_0) and after 5 min. Absorbance unit (AU) values at 5 min were subtracted from AU values at T_0 to determine the change in absorbance. The AU value for the blank sample well was subtracted from the average of the triplicate sample wells to compensate for sample hemolysis, if necessary. The resulting AU values were converted to HEL concentration (mg ml^{-1}) by 4-parameter logistic regression of the standard curve.

Corticosterone analysis

Frozen plasma aliquots were shipped on dry ice to Southeastern Louisiana University, where corticosterone assays were performed. A commercially available enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Inc.) was used to determine plasma corticosterone concentrations (Valverde et al. 1999). Briefly, 1 ml of ethyl ether each time was used to perform two extractions of an 80 µl aliquot of each plasma sample. Aqueous phase was frozen and ether phased containing steroids decanted into a new tube. Ether was fully evaporated under a gentle stream of nitrogen gas in a 37C water bath. Samples were then resuspended in 250 µl of assay buffer. Duplicate 100 µl samples were assayed. A 5-point standard curve, created by serially diluting from 20,000 pg ml^{-1} to 32 pg ml^{-1} of corticosterone, was included in each assay plate. Plates were read at 450 nm in microplate reader (Bio-Rad Model 680). Absorbance data were analyzed with a 4-parameter logistic equation after correcting for blanks. Interassay variation was 11.8%, and mean intraassay variation was 9.5%. All steps except the extraction step were followed according to the manufacturer's protocol. Any samples outside of the range 20–80% bound were assayed again (Clarkson 2016).

Molecular diagnostics for ChHV5 and ChHV6 DNA

Genomic DNA (gDNA) was extracted from thawed whole blood samples using the DNeasy Blood & Tissue kit according to the manufacturer's instructions (Qiagen, Germantown, MD USA). Using a NanoDrop 2000c (Thermo Scientific™, Waltham, MA USA), concentrations of extracted gDNA samples were quantified (units: µg/µl), and gDNA purity was assessed using the ratio of absorbance at 260 nm and 280 nm, with ratios of ~1.8–2.0 considered "pure" (NanoDrop 2007). Extracted DNA samples were assessed for the presence of the ChHV5 UL30 gene segment using a singleplex, probe-based qPCR and the methodologies outlined by Page-Karjian

et al. (2015). Samples were assayed in triplicate, and included in each run of the qPCR assay were 4 no-template controls (nuclease-free distilled water; Qiagen) and a standard curve constructed of 10-fold serial dilutions of a plasmid DNA standard, which also functioned as a positive control. This ChHV5 UL30 standard curve was constructed using a 173 bp fragment amplified with the qPCR primers cloned into the pGEM-T Easy Vector (Promega Life Sciences, Madison, WI USA). The 20 µl qPCR reactions were conducted in 96-well, 0.2 µl PCR plates (Agilent Technologies, Santa Clara, CA USA) containing 10 µl SensiFAST^{TM} Probe Lo-ROX (Bioline, Taunton, MA USA), 0.8 µl (0.32 µM) each of forward and reverse ChHV5 UL30 primers (Integrated DNA Technologies [IDT], Coralville, IA USA), 0.2 µl (0.32 µM) of fluorescent probe (IDT), and 8.2 µl genomic or plasmid DNA (Page-Karjian et al. 2015), according to manufacturer's instructions. All qPCR reactions were carried out using an AriaMx Real-Time PCR System (Agilent) and the following reaction conditions: 10 min at 95°C, and 40 cycles each of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C. Extensive laboratory precautions, as outlined in the MIQE guidelines (Bustin et al. 2009), were taken to avoid assay contamination by genomic or plasmid DNA. Quantitative PCR data were analyzed with the AriaMX qPCR software (Agilent, Version 1.3). Tested samples were considered positive if triplicates had an average copy number equal to or higher than the assay's analytical limit of detection (LOD) of 50 viral copies (Page-Karjian et al. 2015). This relatively low LOD allows us to evaluate samples such as blood that are expected to have low viral copy numbers (Page-Karjian et al. 2015). Using the coefficient of determination (R^2) values, reaction efficiency and precision were calculated for all qPCR assays. To help increase C*q* value accuracy, an adaptive baseline threshold was generated for each assay run via AriaMx software. Regression analysis of the standard curve (Larionov et al. 2005) was used to determine the number of ChHV5 DNA copies for each *C. mydas* DNA sample. ChHV5 UL30 copy numbers were calculated per microgram of gDNA extracted from each biological sample. To demonstrate the presence of amplifiable DNA, primers developed and optimized for *C. mydas* β-actin gene segment (GenBank accession number AY373753.1) were applied to all samples and included in all qPCR runs (after Page-Karjian et al. 2015). To confirm ChHV5 UL30 qPCR assay results, all ChHV5 DNA-positive qPCR products were purified using the QIAquick PCR Purification Kit (Qiagen), 5 µl of 5 µM forward ChHV5 UL30 primer was added to 10 µl of purified amplicon, and shipped on dry ice overnight to Genewiz, where they were sequenced using Sanger sequencing technology (Genewiz, South Plainfield, NJ USA). Resultant sequences obtained were compared to those deposited in the National Center for Biotechnology Information GenBank database using BLAST software (Altschul et al. 1990). Aligned sequences with ≥ 97% identity to the sample sequence were considered a match.

A 1794 bp clone of the ChHV6 DNA polymerase (UL30) gene (clone LETHV 221, GenBank accession number EU006876.1) was used to design a primers/dual-labeled (6-FAM/BHQ1) probe set to detect a unique 112 bp ChHV6 DNA amplicon in submitted samples. Since the virus and a confirmed ChHV6-infected turtle were not available, a 185 bp ChHV6 DNA oligonucleotide, based on EU006876.1, was synthesized (Thermo Life Sciences). Primers complimentary to both ends (Sigma) were used to PCR-generate a double-stranded, 185 bp amplicon. The PCR product was TA cloned (Invitrogen) and used to transform *E. coli* per manufacturer's instructions. Several bacterial colonies were chosen, and each was placed in 200 µl of molecular-grade H_2O after streaking on a lysogeny broth (LB) agarose plate. Total DNA (bacterial and plasmid) was extracted by boiling at 99°C for 10 minutes and centrifuging at 18,407 x *g* for 10 minutes. The DNA was tested using a Roche 480 LightCycler and a probe-based qPCR protocol. ChHV6-positive samples were stored at –80°C for future use as positive controls. Bacteria from a ChHV6-positive DNA sample were grown overnight at 37°C in LB

broth. Aliquots of 150 µl were placed in 850 µl sterile 100% glycerol and frozen at -80°C for future use. Submitted samples were tested with the proprietary qPCR assay (Infectious Disease Laboratory in Athens, Georgia, USA).

ELISA detection of antibodies to ChHV5 and ChHV6 peptides

Separated plasma samples were analyzed for antibodies to ChHV5 and ChHV6 at the Infectious Disease Laboratory (IDL) at the University of Georgia (UGA) College of Veterinary Medicine in Athens, Georgia, USA. Prior to sample analysis, it was noted that the plasma samples contained gelatinous clots and/or flecks of fibrin and were presumed to be adequate for assays validated for turtle serum. To evaluate for exposure to ChHV5, samples were analyzed in triplicate using ELISAs that test for antibodies to ChHV5 and ChHV6 peptide antigens. Both ELISA assays were developed and validated based on modifications of previously published protocols for ChHV5 and ChHV6 assays (Herbst et al. 2008, Coberley et al. 2001a). Synthesized peptides were used for both assays. For ChHV5, the peptide HerbstFibropapGlyh4 referenced in Herbst et al. (2008; CKALKSGKIEGEDRK) was used as antigen. A proprietary peptide, ChHV6GlyH4 was synthesized for the ChHV6 ELISA, based on a unique region in the glycoprotein H gene (GenBank accession number ABX60167.1). Positive serum controls for ChHV5 were obtained from turtles confirmed by histopathology to be positive for FP. Herbst et al. (2008) experimentally confirmed the minimum time to seroconversion after experimental infection with ChHV5. Negative serum controls were obtained from < 7-month-old (post-hatchling) green turtles from a captive population with no history of confirmed FP and shown to be negative for ChHV6GlyH4 antibodies. Positive control sera were obtained by intramuscular injection of the ChHV6GlyH4 peptide (1 mg ml^{-1}) into these same clinically healthy green turtles. These controls are standards developed by the UGA IDL for use in the described commercially available, proprietary assays; they were not developed specifically for this project. There was no cross-reactivity detected between the FP+ and ChHV6GlyH4+ serum samples.

Development of Blood Health Reference Intervals

Blood health reference intervals were determined for study turtles following the consensus guidelines set forth by the American Society for Veterinary Clinical Pathology (Friedrichs et al. 2012). Plasma samples with a 2+ hemolysis score or higher were removed from the dataset for calculation of reference intervals. Next, all remaining analyte data were first tested for Gaussian distribution using the D'Agostino-Pearson test (with $\alpha = 0.05$), and by assessing histograms of reference values for distribution and identification of potential outliers. Data were further evaluated using the Dixon-Reed outlier test to identify outliers at the upper and lower extremities (Horn & Pesce 2003, 2005). The Box-Cox transformation was then used to transform all variables with non-Gaussian distributions, with the exception of potassium, which was transformed using a logarithmic transformation. Due to sample size and the distribution of reference values, the robust method was used to determine all reference intervals (Horn & Pesce 2005, Friedrichs et al. 2012). Mean ± SD, median, range, and central 90% confidence intervals around the upper and lower reference limits were also calculated. Mean ± SD was not calculated for analytes where some values fell below the limits of detection; for reference intervals, values below the limits of detection were assigned to half of the detection limit. All reference interval calculations were performed using MedCalc (MedCalc Software v.18.5, Ostend, Belgium).

Table S1. Summary of mild externally visible abnormalities observed in proportions of adult female green turtles (*Chelonia mydas*).

	N	% of total (N=60)
Mating scar(s)	40	67%
One flipper missing/deformed	6	10%
Small skin laceration(s) on neck	6	10%
Extra/deformed scute(s)	5	8%
Flipper notch(es)	5	8%
Small skin nodule(s)	4	7%
Carapace abnormalities [e.g., crack(s), piece(s) missing, depression(s)]	3	5%
Leech(es) on skin	1	2%
Infected flipper tag	1	2%

Table S2. Significant results of least-squares linear regressions comparing blood health analytes of adult female green turtles (*Chelonia mydas*) to date of nesting season. Equations for the regression lines are provided, in addition to the confidence intervals (CI) of the slopes of the regression lines and the y-intercept. When residuals of the regressions could not be normalized, Spearman's correlations were used.

Analyte	Regression equation	Slope 95% CI	Intercept 95% CI	r^2	*P*	N
Total solids	NA	NA	(–0.688, –0.271)[a]	–0.509[b]	<0.001	51
Phosphorus[c]	y = –0.005x + 208.587	(–0.008, –0.002)	(65.853, 351.321)	0.150	0.005	50
Uric acid[c]	y = –0.049x + 2106.594	(–0.068, –0.030)	(1297.766, 2915.333)	0.362	<0.001	50
Calcium:phosphorus	y = 0.004x – 167.011	(0.001, 0.007)	(–310.053, –23.969)	0.104	0.022	50
Sodium	y = 0.107x – 4425.117	(0.055, 0.158)	(–6617.484, –2232.750)	0.264	<0.001	51
Chloride	y = 0.109x – 4557.400	(0.056, 0.161)	(–6812.457, –2302.344)	0.261	<0.001	51
Total protein[d]	y = –0.216x + 9330.592	(–0.357, –0.075)	(3278.552, 15382.633)	0.165	0.003	50
Albumin:globulin	y = –0.002x + 65.463	(–0.003, –0.001)	(22.376, 108.550)	0.158	0.004	51
Pre-albumin[c]	y = 0.004x – 158.462	(0.001, 0.007)	(–281.564, –35.360)	0.122	0.012	51
Albumin[c]	y = –0.016x + 702.828	(–0.022, –0.011)	(454.517, 951.139)	0.395	<0.001	51
$Alpha_1$-globulins[c]	y = –0.003x + 147.093	(–0.006, –0.0004)	17.557, 276.628)	0.094	0.029	51
$Alpha_2$-globulins[c]	y = –0.008x + 342.603	(–0.013, –0.003)	(118.701, 566.506)	0.150	0.004	51
Total globulins[c]	y = –0.010x + 405.048	(–0.018, –0.0003)	(22.922, 787.174)	0.084	0.041	50

[a]95% confidence interval for Spearman's rho
[b]Spearman's rho (r_s)
[c]Analyte was square-root transformed
[d]Outliers removed using Tukey's method

Table S3. Descriptive statistics for plasma analytes that measure innate immune capacity (lysozyme,), oxidative stress (catalase, total glutathione, ROS/RNS), and physiological stress (corticosterone) in adult female green turtles (*Chelonia mydas*) nesting in Juno Beach, Florida.

	Units	N	Mean ± SD	Median	Range
Lysozyme	mg HEL ml^{-1}	45	3.25 ± 3.86	1.73	0.27–18.05
Catalase	nmol ml min^{-1}	57	60.17 ± 45.33	54.80	1.01–204.82
Total glutathione	nmol ml min^{-1}	58	5855 ± 2357	5466	1526–12,529
Superoxide dismutase	U ml^{-1}	59	29.84 ± 7.77	28.88	15.27–53.75
ROS/RNS	nM	59	1468 ± 1932	770	202–12718
Corticosterone	nmol l^{-1}	55	7.8 ± 6.48	5.89	2.08–38.89

Table S4. Spearman's correlation coefficient analysis between haptoglobin concentrations and PCV, and plasma electrophoresis analytes in adult female green turtles (*Chelonia mydas*) nesting in Juno Beach, Florida.

	Correlation coefficient (R)	2-tailed p-value
PCV	–0.02	0.91
Prealbumin	0.05	0.72
Albumin	0.28*	0.03
$Alpha_1$	0.23	0.09
$Alpha_2$	0.02	0.86
Beta	0.8*	<0.001
Gamma	0.27	0.09
A:G	–0.36*	0.01
Total globulin	0.62*	<0.001
Total protein	0.54*	<0.001

* Denotes statistically significant correlations.

Table S5. Plasma ROS/RNS concentration, antioxidant enzyme activity, innate immunity, and physiological stress (corticosterone) measurements in various sea turtle species at different life stages, as presented in the scientific literature to date and compared to data from this study.

Analyte	Units	Species	Year(s)	Activity	*n*	Mean ± SD	Reference
ROS/RNS	nM	*C. caretta*	2015	Nesting	36	2898 ± 2169	Perrault et al. 2017b
		C. caretta	2014	Nesting	33	4046 ± 3072	Perrault et al. 2016
		L. kempii	2014	Foraging	24	535.74 ± 501.53	Perrault et al. 2017a
		C. mydas	2014	Foraging	9	187.13 ± 191.99	Perrault et al. 2017a
		C. mydas	2017	Nesting	55	1464 ± 1938	This study
SOD	U ml^{-1}	*C. caretta*	2015	Nesting	36	92.5 ± 58.6	Perrault et al. 2017b
		C. caretta	2014	Nesting	47	169 ± 83	Perrault et al. 2016
		L. kempii	2011–2013	Foraging	13	48.0 ± 10.3	Perrault et al. 2014
		L. kempii	2014	Foraging	24	25.83 ± 10.29	Perrault et al. 2017a
		C. mydas	2014	Foraging	10	14.95 ± 4.12	Perrault et al. 2017a
		C. mydas	2017	Nesting	55	30.12 ± 7.74	This study
Lysozyme	µg HEL ml^{-1}	*C. caretta*	2000–2001	Foraging	48	6.58 ± 0.58*	Keller et al. 2006
		C. caretta	2015	Nesting	24	5.0 ± 2.5	Perrault et al. 2017b
		C. caretta	2001	Foraging	12	4.96 ± 0.6*	Day et al. 2007
		C. caretta	2003	Foraging	58	11.02 ± 0.82*	Day et al. 2007
		C. caretta	2014	Nesting	47	5.2 ± 2.0	Perrault et al. 2016
		L. kempii	2014	Foraging	24	3.97 ± 1.41	Perrault et al. 2017a
		C. mydas	2014	Foraging	10	3.19 ± 1.56	Perrault et al. 2017a
		C. mydas	2017	Nesting	41	3.47 ± 3.97	This study
Haptoglobin	mg ml^{-1}	*C. caretta*	2008–2012	Rehabilitating	18	0.27[a] (0.05–0.36)[b]	Dickey et al. 2014
		C. mydas	2017	Nesting	52	1.24 ± 0.41	This study
Corticosterone[d]	ng ml^{-1}	*D. coriacea*	1996–1998	Nesting	32	1.4–3.9[b]	Rostal et al. 2001
		C. caretta	2015	Nesting	11	1.93[a](0.47– 5.34)[b]	Flower et al. 2015
		C. caretta	2001, 2003	Foraging	37	11.02 ± 0.82*	Day et al. 2007
		C. caretta	2009	Nesting	6	0.1 ± 0.09	Sozbilen & Kaska 2018
		L. olivacea	1991	Nesting	10	0.33	Valverde et al. 1999
		N. depressus	2004	Nesting	20	0.41[c] ± 0.05*	Ikonomopoulou et al. 2014
		C. mydas	2004	Nesting	14	0.19[c] ± 0.06*	Ikonomopoulou et al. 2014
		C. mydas	2002	Nesting	22	0.42 ± 0.04*	Al-Habsi et al. 2006
		C. mydas	1997	Nesting	23	2.81 ± 0.18	Hamann et al. 2002a
		C. mydas	2017	Nesting	55	2.25 ± 1.87	This study