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Mitogen-induced lymphocyte proliferation in loggerhead sea turtles: comparison of methods and effects of gender, plasma testosterone concentration, and body condition on immunity

Jennifer M. Keller[a,*], Patricia D. McClellan-Green[a,b], A. Michelle Lee[c,d,e], Mike D. Arendt[f], Philip P. Maier[c,f], Al L. Segars[c,f], J. David Whitaker[c,f], Deborah E. Keil[e,1], Margie M. Peden-Adams[c,d,e]

[a]*Coastal Systems Science and Policy, and Integrated Toxicology Program, Nicholas School of the Environment and Earth Sciences, Duke University, 135 Duke Marine Lab Road, Beaufort, NC 28516, USA*

[b]*Department of Environmental and Molecular Toxicology, and Center for Marine Science and Technologies, North Carolina State University, Morehead City, NC 28557, USA*

[c]*College of Charleston, Grice Marine Laboratory, 205 Fort Johnson Road, Charleston, SC 29412, USA*

[d]*Department of Pediatrics, Medical University of South Carolina, 221 Fort Johnson Road, Charleston, SC 29412, USA*

[e]*Marine Biomedicine and Environmental Science Center, Medical University of South Carolina, 221 Fort Johnson Road, Charleston, SC 29412, USA*

[f]*South Carolina Department of Natural Resources, 217 Fort Johnson Road, Charleston, SC 29412, USA*

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Abstract

A fully functioning immune system is vital to the survival of threatened and endangered sea turtles. Immunological protection against diseases in any organism can be reduced by a number of natural and anthropogenic factors, such as seasonal changes, malnutrition, disease states, and contaminant exposure. These factors are even more critical when they occur in endangered species or populations. To identify alterations in the immunological health of loggerhead sea turtles (*Caretta caretta*), the mitogen-induced lymphocyte proliferation (LP) assay was developed using peripheral blood leukocytes (PBLs). Collection and culture conditions were optimized for this assay using non-lethal blood samples collected from free-ranging turtles along the southeastern US coast. During the collection, two anticoagulants (sodium heparin and lithium heparin) were compared to determine effects of different ions on assay results. Optimal culture conditions were established for loggerhead PBLs while two different methods of measuring LP were compared: (1) the traditional radioactive ^{3}H-thymidine assay and (2) a non-radioactive, colorimetric method utilizing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT). The results indicate that the ^{3}H-thymidine and the non-radioactive MTT methods did not correlate with each other and that the use of

Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium; SI, stimulation index

* Corresponding author. Present address: National Institute of Standards and Technology, Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412, USA Tel.: +1 843 762 8863; fax: +1 843 762 8742.

E-mail address: jennifer.keller@noaa.gov (J.M. Keller).

[1] Present address: National Institute of Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505, USA.

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heparin type did not influence the results of the LP assay. Lastly, using these optimized methods, we investigated the effect of gender, plasma testosterone concentration, and body condition on LP in loggerhead turtles and found that none of the parameters largely influenced LP.
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Keywords: Turtle; Mitogen; Lymphocyte proliferation; Nutrition; Sex differences; Steroids

1. Introduction

While the general fundamentals of the reptilian immune system are known, the immune functions of threatened and endangered sea turtles have not been well characterized. A few studies on immune function in sea turtles are available, but these focus on the green sea turtle (*Chelonia mydas*), a species that is heavily afflicted by fibropapillomatosis. Turtles with this disease are affected with non-cancerous tumors often found on the skin that can fatally affect mobility and foraging (Work et al., 2001). Green turtles with fibropapillomas, in both Florida and Hawaii, have exhibited compromised immunological function compared to disease-free turtles, as evidenced by their decreased mitogen-induced lymphocyte proliferation (LP) response (Cray et al., 2001; Lutz et al., 2001; Work et al., 2001). The LP assay has been optimized in green sea turtles by McKinney and Bentley (1985), but it has not been used to assess the immune function of other sea turtle species.

The loggerhead sea turtle (*Caretta caretta*) is considered a threatened species under the US Endangered Species Act due to past and current population declines (TEWG, 2000). It is also afflicted with several diseases, including fibropapillomatosis. In addition, loggerhead turtles accumulate higher concentrations of organochlorine contaminants than green sea turtles (Keller et al., 2004). Organochlorine contaminants have been associated with increased incidences of immunosuppression in fish, birds, and marine mammals thus subjecting these species to increased risk of infections or invasion by foreign pathogens (Grasman et al., 1996; Ross et al., 1996; Arkoosh and Collier, 2002). Unfortunately, immunotoxicological effects of contaminants have not been thoroughly investigated in reptiles, much less in sea turtles. Because a healthy immune system directly relates to the survival of individuals and the stability or recovery of sensitive populations, it is important to assess sea turtle immune responses in order to understand how they may be modulated by natural or anthropogenic factors.

This study describes the optimal conditions for performing the LP assay in loggerhead sea turtles. Using paired samples, we compared two methods of measuring LP, the ^{3}H-thymidine assay and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT) assay. The traditional method measures the incorporation of ^{3}H-thymidine into cellular DNA of cells in the S phase of cell division during the last hours of the experimental culture. The non-radioactive, colorimetric method utilizing MTT has been proposed as an alternative method for measuring LP (Mosmann, 1983). This method measures the viability of cells during the last hours of culture by quantifying the conversion of MTT by mitochondrial dehydrogenases into a blue-colored formazan product. Because this method eliminates costs associated with purchasing and disposing of radioactivity and negates the need for expensive cell harvesters and scintillation counters, it is an advantageous alternative for many researchers. However, certain studies have shown that the results obtained using the MTT assay differ from those with the ^{3}H-thymidine assay and that they may not correlate (Reubel and Bauerfeind, 1989; Chen et al., 1990; Espelid et al., 2003). Therefore, it is important to determine the usefulness of this alternative method for each species and sample type. Following the optimization of the methods, we examined the role of gender, plasma testosterone concentrations, and body condition of loggerhead turtles on the LP responses.

2. Materials and methods

2.1. Sampling

Turtles for this study were captured during two ongoing tagging studies. Twenty-three free-ranging

juvenile loggerhead sea turtles with straight carapace lengths (SCL was measured from the nuchal notch to the most marginal posterior notch) between 49 and 77.3 cm were captured as by-catch from a pound net fishery located in Core Sound, North Carolina (NC), USA in July 2001. These turtles were used for the comparison between ^{3}H-thymidine and MTT methods. Blood samples were collected within 15 min of capture from the dorsocervical sinus using double-ended needles directly into blood collection tubes containing sodium heparin (Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ). For the comparison between anticoagulants, 13 free-ranging juvenile loggerhead sea turtles with SCL between 56.1 and 82.7 cm were captured in offshore waters of Charleston, South Carolina (SC) and in Core Sound, NC, USA from late May to the middle of June 2003. Blood samples were collected from the dorsocervical sinus using double-ended needles into a tube containing sodium heparin and a second tube containing lithium heparin (Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ). A total of 298 loggerhead turtles were captured from offshore waters of SC, Georgia (GA), and northern Florida (FL) during the summer months of 2001–2003. Blood samples were collected from a portion of these turtles for LP, and when possible, the turtles were measured and weighed. The LP responses of these turtles were used to examine the influence of gender, plasma testosterone, and body condition on the LP response. Blood was kept cool either on ice or in a refrigerator (4 °C) until processed for LP within 36 h of collection. Turtles collected in SC, GA, and FL waters were randomly captured in 8 in. trawl nets without turtle excluder devices using a trawl tow time of 30 min. All turtles were released near their capture location. Body condition was calculated as weight (kg) divided by the cube of SCL from the nuchal notch to the most posterior marginal notch (cm) and multiplied by 100,000 [body condition = kg/(SCL^{3}) $\times$ 100,000] as described in Bjorndal et al. (2000). Sex of the turtles was determined by measuring plasma testosterone concentrations (Owens, 1997).

2.2. Optimization of lymphocyte proliferation

In order to optimize the lymphocyte proliferation assay for use with loggerhead sea turtle peripheral blood, various conditions were tested including several T- and B-cell mitogens at numerous concentrations, multiple incubation time points (1–12 days), varied cell densities (0.125×10^{5}, 0.25×10^{5}, 0.5×10^{5}, 1×10^{5}, 1.8×10^{5}, 3×10^{5}, 4×10^{5}, 5×10^{5}, and 1×10^{6} cells/well), diverse media preparations (different lots of FBS, different concentrations of FBS, different concentrations of sea turtle plasma rather than FBS, with and without the addition of NaCl, with and without the addition of HEPES, with and without the addition of EDTA), various pH levels (6.9, 7.4, and 7.7), two incubation temperatures (25 and 30 °C), and two different cell isolation techniques (the cell isolation method described herein was compared to a method utilizing Percoll described in Harms et al. (2000)). Samples from animals collected in Core Sound, NC were used for optimization of the MTT assay. Samples from NC, SC, GA, and FL were used to optimize the ^{3}H-thymidine method.

2.3. Comparison of ^{3}H-thymidine and MTT methods

2.3.1. ^{3}H-thymidine assay

Blood samples for the ^{3}H-thymidine assay were sent overnight with frozen ice packs to the Medical University of South Carolina and processed on the day following collection. PBLs were collected from the buffy coat (the layer of PBLs between the plasma and RBCs) using a slow spin technique. The tubes were centrifuged at 500 rpm ($42 \times g$) in a swinging bucket rotor for 25 min at 8 °C. The PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 1500 rpm ($377 \times g$) for 8 min, the plasma was removed and the cell pellet was gently resuspended in 1 ml of RPMI 1640 media (Mediatech, Inc., Herndon, VA) supplemented with 5% FBS (BioWhittaker, Walkersville, MD lot # 8S006F), 1% (v/v) non-essential amino acids (Gibco, Grand Island, NY), 1 mM sodium pyruvate (Gibco, Grand Island, NY), 10 mM HEPES (Mediatech, Inc., Herndon, VA), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Mediatech, Inc., Herndon, VA), which was initially brought to pH 6.9. To remove any residual plasma, the cells were centrifuged again at 1500 rpm ($377 \times g$) for 8 min, the supernatant was removed, and the cells were resuspended in 1 ml of media. The number of

viable PBLs was determined via trypan blue exclusion using a hemacytometer. All counts were performed by one person to ensure consistency. Cells were diluted with media and plated at a density of 1.8×10^5 cells/well into 96 well plates.

Mitogens chosen to stimulate T-cells were phytohemagglutinin P (PHA; Amersham Pharmacia Biotech Inc., Pascataway, NJ) and concanavalin A (ConA) type IV from Jack Bean (*Canavalia ensiformis*) (Sigma, St. Louis, MO). Mitogens chosen that stimulate B-cells were lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8 (Sigma, St. Louis, MO) and phorbol 12,13-dibutyrate (PDB; Sigma, St. Louis, MO). PDB was previously shown to stimulate avian B-cells (Scott and Savage, 1986). Mitogens were diluted in media and 50 μl were added to each well. Each control (media only) and mitogen concentration was tested in triplicate wells with a final volume of 100 μl/well. Cells were incubated at 30 °C with 5% CO_2. Following a 5-day incubation (120 h), 0.5 μCi/well of ^{3}H-thymidine (ICN Biomedicals, Inc., Irvine, CA) was added in a volume of 100 μl media to each well. Plates were further incubated for 16 h and then harvested onto Unifilter plates (Packard, Meridian, CT) using a Packard Filtermate 96-well plate harvester, and the plates were allowed to dry. Once dry, 25 μl of Microscint 20 (Packard, Meridian, CT) was added to each well, and the samples were analyzed using a Packard Top Count-NXT scintillation counter. Stimulation index (SI) was calculated as the cpm of mitogen-stimulated cells divided by the cpm of unstimulated control (media only) cells.

2.3.2. MTT assay

In order to compare LP measured by the ^{3}H-thymidine assay to the non-radioactive, colorimetric MTT assay, 23 paired loggerhead blood samples were measured for LP using both techniques. Sample collection, cell preparation, and culture conditions were identical to those used for the ^{3}H-thymidine assay except for the following minor changes. PBLs were collected using the slow spin technique described above, but the cells were isolated, counted, and plated on the same day as blood collection. The media used for the MTT assay was identical to the media used in the ^{3}H-thymidine assay, except for the manufacturer of non-essential amino acids (Mediatech, Inc., Herndon, VA), sodium pyruvate (BioWhittaker, Walkersville, MD) and HEPES (BioWhittaker, Walkersville, MD). Each control (media only) and mitogen concentration was tested in triplicate wells with a final volume of 100 μl/well using 1.8×10^5 cells/well. The same mitogen types, lots, and concentrations were used in both methods. On day 5 of mitogen exposure (116 h), cell proliferation was measured using CellTiter 96, a non-radioactive cell proliferation assay employing MTT (Promega, Inc., Madison, WI). The dye solution was added to each well (15 μl/well) and incubated for 4 h. The solubilization solution was added (100 μl/well) to stop the cellular conversion of the MTT dye. Plates were placed at 30 °C for 15 h to allow the formazan product to completely solubilize. Absorbance was read at 570 and 650 nm on a spectrophotometric plate reader (Molecular Devices Spectramax 190, Sunnyville, CA). Absorbance of the product at 650 nm was subtracted from the absorbance at 570 nm ($Abs_{570–650}$) to calculate total conversion of dye. Furthermore, $Abs_{570–650}$ was corrected by subtracting the background absorbance of wells that contained only media, mitogens, and the MTT kit reagents without cells. The SI was calculated as the corrected absorbance of mitogen-stimulated cells divided by the corrected absorbance of unstimulated, control (media only) cells.

2.4. Optimized LP assay for examining the influence of anticoagulant, gender, plasma testosterone concentration, and body condition

LP was measured using slight modifications of the ^{3}H-thymidine method described above. These modifications represent the optimized conditions that generally resulted in the highest stimulation indices. Briefly, PBLs were isolated from the buffy layer using the slow spin technique within 36 h of blood collection. Cells were diluted in RPMI 1640 media (Mediatech, Inc., Herndon, VA) supplemented with 5% FBS (Hyclone, Logan, UT was used in 2001 and 2002; and Gemini, Calabasas, CA was used in 2003; experiments with paired samples in 2003 indicated that this change did not affect LP results), 1% (v/v) non-essential amino acids (Gibco, Grand Island, NY), 1 mM sodium pyruvate (Gibco, Grand Island, NY), 10 mM HEPES (Mediatech, Inc., Herndon, VA), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Mediatech, Inc., Herndon, VA), which was initially brought to pH 6.9. Cells were plated at 1.8×10^5 cells/

well in a final volume of 200 μl/well. Mitogens and final concentrations chosen were PHA P (Sigma, St. Louis, MO) at 5 μg/ml, ConA (type IV-S) at 20 μg/ml, LPS (*E. coli* 0111:B4) at 10 μg/ml, and PDB at 0.2 μg/ml. Cells were incubated at 30 °C with 5% CO_2. For the comparison between sodium and lithium heparin, all mitogens were tested on day 5 of incubation. For the gender, testosterone, and body condition comparisons, PHA and ConA were tested on day 5 of incubation, and LPS and PDB were tested on day 4. Incubation with ^{3}H-thymidine, cell harvesting, and analysis were identical to the method described above.

2.5. Statistics

All statistical analyses were performed using Systat 10.0 (SPSS Inc., Chicago, IL) or JMP 4.0.2 (SAS Institute, Cary, NC). Non-parametric tests were used, because most LP responses were not normally distributed. The Wilcoxon signed rank test was used on paired data to test differences between the ^{3}H-thymidine and MTT methods, to test differences between the two heparin types, to test two different cell densities, and to test two different cell isolation techniques. A paired sample t-test was used to examine differences between media compositions with different pH because the responses in this experiment were normally distributed. Spearman rank correlations were used to examine correlations between the ^{3}H-thymidine and MTT methods, between testosterone and LP, and between body condition and LP. LP responses were also examined for gender and age (juveniles versus adults) differences (adults were identified as turtles greater than 85 cm SCL) using the Kruskal–Wallis test.

3. Results

3.1. Optimization of lymphocyte proliferation

Mitogens tested included PHA (1–500 μg/ml), ConA (1–500 μg/ml), LPS (2.5–800 μg/ml), and PDB (0.05–1.0 μg/ml) over several days of incubation.

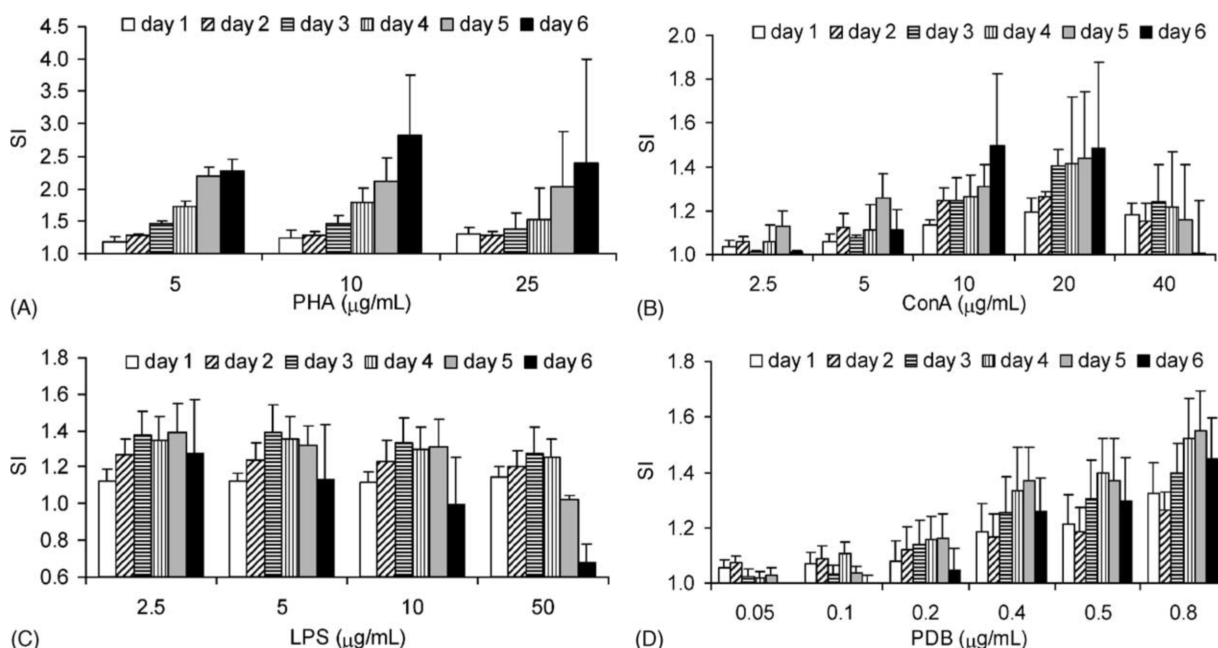

Fig. 1. Optimization of mitogen-induced proliferation of lymphocytes obtained from loggerhead sea turtles measured by the non-radioactive MTT assay using increasing concentrations of two T-cell mitogens (A and B) and two B-cell mitogens (C and D) at several incubation time points. Data are reported as mean and S.E.M of three turtles. Cells were plated at 1.8×10^5 cells/well. SI: stimulation index (absorbance from stimulated wells/absorbance from unstimulated wells). Mean (S.E.M.) corrected absorbance values for the unstimulated cells were 0.171 (0.044), 0.172 (0.042), 0.157 (0.040), 0.122 (0.030), 0.126 (0.032), and 0.124 (0.030) for days 1, 2, 3, 4, 5, and 6, respectively.

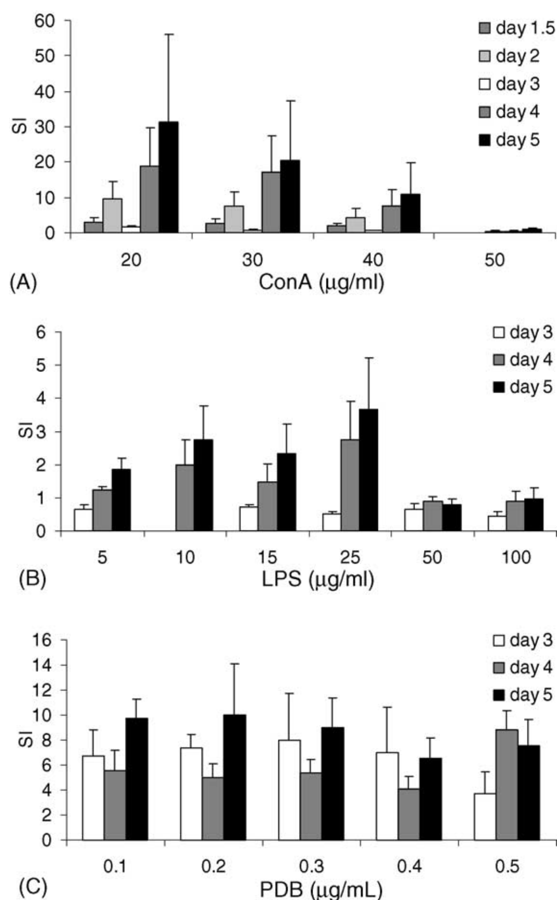

Fig. 2. Optimization of mitogen-induced proliferation of lymphocytes obtained from loggerhead sea turtles measured by the ^{3}H-thymidine assay using increasing concentrations of a T-cell mitogen (A) and two B-cell mitogens (B and C) at several incubation time points. Data are reported as mean and S.E.M. of all turtles tested in these experiments. Sample sizes ranged from 2 to 24 depending on the mitogen concentration and time point. Cells were plated at 1.8×10^5 cells/well. SI: stimulation index (cpm from stimulated wells/cpm from unstimulated wells). Mean (S.E.M.) cpm values for the unstimulated cells were 401 (103), 191 (36), 375 (72), 127 (19), and 100 (17) for days 1.5, 2, 3, 4, and 5, respectively.

Optimum conditions for the assay are discussed, but due to the enormity of the data, not all data are shown. Example graphs of mitogen concentrations and incubation time points are shown for the MTT assay (Fig. 1) and the ^{3}H-thymidine assay (Fig. 2). The optimum mitogen concentrations for the MTT assay were 10 μg/ml PHA, 20 μg/ml ConA, 2.5 μg/ml LPS, and 0.8 μg/ml PDB (Fig. 1). These concentrations were similar to the optimal concentrations for the ^{3}H-thymidine assay, except PDB resulted in the optimal response in the range of 0.1–0.2 μg/ml and LPS at 25 μg/ml using ^{3}H-thymidine (Fig. 2). Five to six days resulted in the highest SI for the MTT assay (Fig. 1). The optimum time point varied between 4 and 5 days for individual animals using the ^{3}H-thymidine method, and 5 days resulted in the overall combined highest SI. Cell density was found to be optimal at 1.8×10^5 cells/well for three mitogens. For example, the respective mean stimulation indices (SEM) for 1.0×10^5 cells/well and for 1.8×10^5 cells/well were 1.7 (1.1) and 5.3 (2.0) for 20 μg/ml ConA; 1.1 (0.5) and 4.2 (2.5) for 25 μg/ml LPS; and 9.1 (1.8) and 9.7 (1.6) for 0.1 μg/ml PDB using paired samples from five turtles incubated for 5 days and measured by the ^{3}H-thymidine assay. The only statistically significant difference between cell densities, however, occurred with LPS (Wilcoxon signed rank test; $p < 0.05$). The initial pH of the media was tested using mononuclear cells isolated at the interface of 45 and 55% Percoll layers as described in Harms et al. (2000) from three paired samples, plated at 1×10^5 cells/well, and incubated for 4 days. Proliferation was measured by the MTT assay. The respective mean stimulation indices (SEM) for pH 7.7 and pH 6.9 were 1.23 (0.19) and 1.24 (0.10) for 10 μg/ml PHA; 1.43 (0.14) and 1.56 (0.14) for 10 μg/ml ConA; and 1.15 (0.04) and 1.34 (0.03) for 20 μg/ml LPS. The initial pH of the media had a small influence on the LP responses with pH 6.9 generally resulting in slightly higher responses, but only the LPS response exhibited a statistically significant difference between pH levels (paired sample *t*-test; $p < 0.05$).

The influence of supplementing the media with loggerhead sea turtle serum and plasma was also assessed. Sea turtle serum clotted in the culture wells and prevented meaningful measurement of LP. A range of concentrations of sea turtle plasma was compared to 10% FBS using the MTT assay (Fig. 3). Plasma was obtained and mixed from four turtles, and the mixture was tested on cells isolated from three of these turtles. Sea turtle plasma influenced the PHA response in a variety of ways depending on the individual turtle; it had no effect on the LP response of cells from turtle ID 3-47, an inhibitory effect on cells from turtle ID 3-48, and a stimulatory effect on cells from turtle ID 3-49 (Fig. 3A). Sea turtle plasma inhibited the LPS stimulation response in cells from all three turtles (Fig. 3B). Overall, these results suggested that FBS was preferred over sea turtle

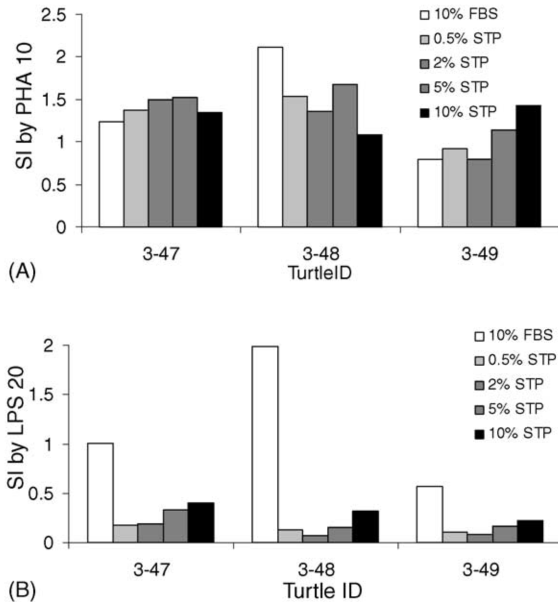

Fig. 3. Comparison of the effect of media supplemented with fetal bovine serum (FBS) or sea turtle plasma (STP) on mitogen-induced proliferation of lymphocytes obtained from three loggerhead sea turtles measured by the MTT assay using 10 μg/ml PHA (A) and 20 μg/ml LPS (B). SI: stimulation index (absorbance from stimulated wells/ absorbance from unstimulated wells). Cells were plated at 1×10^5 cells/well and incubated for 4 days. Mean (S.E.M.) corrected absorbance values for the unstimulated cells were 0.039 (0.017) for 10% FBS, 0.031 (0.012) for 0.5% STP, 0.053 (0.022) for 2% STP, 0.033 (0.008) for 5% STP, and 0.024 (0.007) for 10% STP.

plasma. During early optimization studies with the ^{3}H-thymidine assay, small amounts of turtle plasma remaining in the cell suspension dampened the proliferation response and made cell harvesting difficult. Thus, in the optimized methods, PBLs were washed twice with media to remove all plasma.

Additionally, the effect of different techniques to isolate cells was also compared. Cells from eight turtles were isolated with both the slow spin technique as described in the methods section or the method described by Harms et al. (2000), in which the cells were isolated from the interface of a 45 and a 55% discontinuous Percoll gradient that was layered under a suspension of cells taken from the buffy layer. Cells were plated at 1×10^5 cells/well, incubated for 4 days, and tested for proliferation using the MTT assay. The isolation technique did not affect the response to 50 μg/ml LPS (mean stimulation indices [SEM] were 1.04 [0.26] for the slow spin technique versus 1.20 [0.21] for the Percoll method), but the response to 5 μg/ml PHA was significantly stronger in the cells that were isolated from the slow spin technique (2.61 [0.63]) compared to the Percoll technique (1.59 [0.26]) (Wilcoxon signed rank test; $p < 0.05$).

3.2. Comparison of ^{3}H-thymidine and MTT methods

The optimum assay conditions based on the results shown above were chosen for the comparison of MTT and ^{3}H-thymidine methods. Paired samples from turtles captured in Core Sound, NC were used for this comparison. Spearman rank correlations were used to determine relationships between the two methods. No significant correlations were observed between the two methods for any of the mitogens tested (all p-values > 0.05). Spearman rank correlation coefficients (Rs) were 0.456 for 5 μg/ml PHA, 0.127 for 20 μg/ml ConA, 0.341 for 2.5 μg/ml LPS, and 0.091 for 0.4 μg/ml PDB.

The mean SI measured using MTT was consistently 1.65 or lower regardless of the mitogen type or concentration (Fig. 4). The traditional radioactive ^{3}H-thymidine assay resulted in significantly higher SI than the non-radioactive MTT assay for PHA, LPS, and PDB. No significant difference between methods was observed for ConA, and the response to this T-cell mitogen was low using both methods. The lower responses measured by the MTT assay, together with the lack of correlation between the two methods, suggest that the MTT method may not be as sensitive in this cell system as the ^{3}H-thymidine assay for detecting slight changes in immune function of loggerhead sea turtles.

3.3. Comparison of sodium heparin to lithium heparin

The effects of sodium and lithium heparin on the outcome of the LP assay were assessed in order to determine whether the salt-type of the anticoagulant played a role in the immune response. Cells were plated at 1.8×10^5 cells/well and incubated for 5 days. Proliferation was measured by the ^{3}H-thymidine assay in 13 turtles for PHA, LPS, and PDB and in nine turtles for ConA. Cells collected in sodium and lithium salts, respectively, resulted in mean stimulation indices (SEM) of 352 (77) and 329 (93) for 5 μg/ml PHA; 41.4

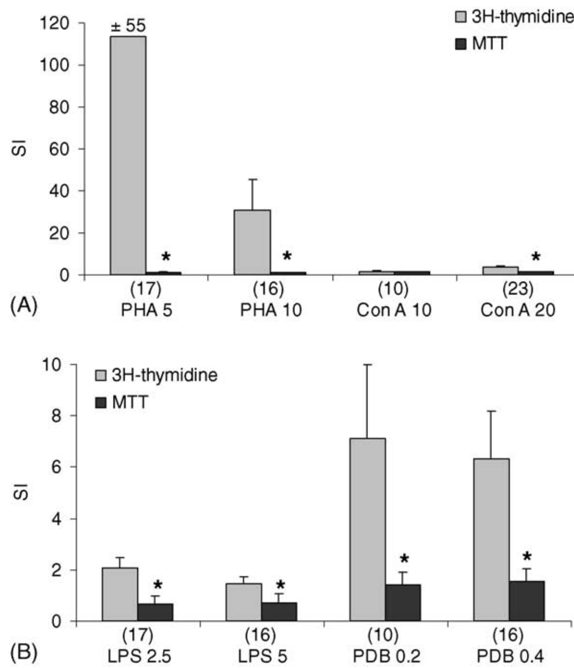

Fig. 4. Mitogen-induced proliferation of lymphocytes obtained from loggerhead sea turtles measured by the traditional ^{3}H-thymidine assay and the non-radioactive MTT assay using two T-cell mitogens (A) and two B-cell mitogens (B). Cells were plated at 1.8×10^{5} cells/well and incubated for 5 days. Sample sizes are in parentheses. Numbers next to mitogen names represent the final concentration (μg/ml) of mitogen in the culture wells. SI: stimulation index (value from stimulated wells/value from unstimulated wells). Mean (S.E.M.) proliferation values for the unstimulated cells of all 23 samples were 148 (58) cpm in the ^{3}H-thymidine assay and 0.166 (0.009) corrected absorbance in the MTT assay. Data are reported as mean and SEM. *An asterisk denotes a difference between the two methods (Wilcoxon signed rank test; $p < 0.05$).

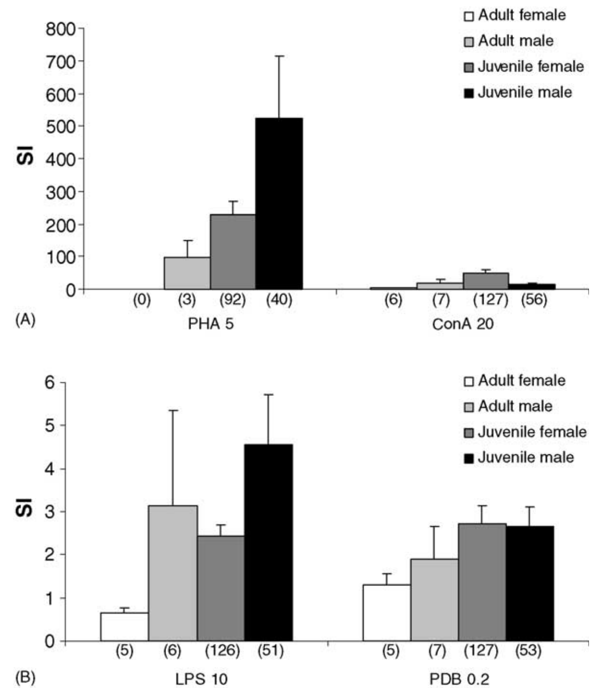

Fig. 5. Comparison of mitogen-induced proliferation of lymphocytes obtained from female and male and juvenile and adult loggerhead sea turtles measured by the ^{3}H-thymidine assay using two T-cell mitogens (A) and two B-cell mitogens (B). Turtles were captured in offshore waters of South Carolina, Georgia, and northern Florida in the summers of 2001–2003. Sample sizes are in parentheses. Cells were plated at 1.8×10^{5} cells/well and were incubated in LPS and PDB for 4 days and in PHA and ConA for 5 days. Numbers next to mitogen names represent the final concentrations (μg/ml) of mitogen in the culture wells. SI: stimulation index (cpm from stimulated wells/cpm from unstimulated wells). Data are reported as mean and S.E.M. No significant differences between groups were detected (Kruskal–Wallis test; $p > 0.05$).

(17.0) and 58.3 (25.5) for 20 μg/ml Con A; 6.65 (2.81) and 5.70 (2.33) for 10 μg/ml LPS; and 5.27 (1.72) and 1.98 (0.79) for 0.2 μg/ml PDB. Although the sodium heparin produced slightly higher SI for the B-cell mitogen, PDB, there was no statistically significant differences between the SI for the two anticoagulants (Wilcoxon signed rank test).

3.4. Influence of gender, plasma testosterone, and body condition on LP response

Gender of the turtles did not significantly influence the LP response of either the juveniles or the adults (adults were identified as turtles greater than 85 cm SCL) captured in offshore waters of SC, GA, and northern FL during the summers of 2001–2003 (Fig. 5). Although there appeared to be a stronger response of males to PHA and LPS, there were no statistically significant differences between sexes for any of the mitogens tested (all p-values > 0.05).

The influence of plasma testosterone concentration on the LP response was examined for four groups of turtles (juvenile females, adult females, juvenile males, and adult males). The turtle size classes were separated because testosterone concentrations are known to differ among these groups (reviewed in Owens, 1997). Juvenile male sea turtles have

Table 1
Spearman rank correlation coefficients (Rs) and *p*-values for correlations between plasma testosterone concentrations and mitogen-induced lymphocyte proliferation responses in loggerhead sea turtles

Mitogen[a]	Adult females[b]			Juvenile females			Adult males[b]			Juvenile males		
	n	Rs	*P*	*n*	Rs	*P*	*n*	Rs	*P*	*n*	Rs	*P*
PHA5	0	NA	NA	92	0.160	0.128	3	0.500	0.667	40	0.084	0.607
ConA 20	6	−0.486	0.329	127	0.156	0.079	7	0.107	0.819	56	0.025	0.853
LPS10	5	−0.700	0.188	126	−0.101	0.262	6	0.657	0.156	51	0.068	0.635
PDB 0.2	5	−0.700	0.188	127	−0.308	0.000	7	0.214	0.645	53	0.005	0.970

n: Sample size; NA: data not available.
[a] Numbers next to mitogen names represent the final concentration (μg/ml) of mitogen in the culture wells.
[b] Adults were identified as turtles greater than 85 cm straight carapace length measured from the nuchal notch to the most posterior marginal notch.

significantly higher plasma testosterone concentrations than juvenile females. Adult females have low concentrations similar to juvenile females unless they are nesting. During the nesting season, adult female testosterone concentrations increase and in some instances can be greater than some males. As male sea turtles mature their testosterone concentrations generally increase, but as juveniles the concentration of testosterone is positively correlated with temperature whereas in adult males it correlates with the reproductive cycle. The adult males sampled in the current study had higher testosterone concentrations than the juvenile males, thus these groups were separated (Wilcoxon *t*-test, $p = 0.0003$; data not shown). Spearman rank correlations were used to compare testosterone concentrations to the LP responses. Only one correlation was statistically significant, in which testosterone concentrations were negatively, but weakly, correlated with the PDB response in juvenile females (Table 1).

LP responses of the turtles were also compared to their nutritional status as measured by body condition. Mean (S.D.) body condition measured from 245 turtles was 16.0 (1.7) and ranged from 11.0 to 22.5. A subset of these turtles was tested for LP, and Spearman rank correlations were assessed between their LP responses and their body condition indices. No significant correlations were observed for any of the four mitogens (Table 2). To further investigate these correlations, the LP responses measured from all turtles captured in offshore waters of SC, GA, and northern FL are shown in Fig. 6. Four turtles with the lowest body condition indices and three turtles observed with sunken plastrons, a common sign of emaciation, are also shown for comparison. The LP responses of these thin turtles did not consistently fall within the low end of the LP responses.

Table 2
Spearman rank correlation coefficients (Rs) and *P*-values for correlations between body condition and mitogen-induced lymphocyte proliferation responses in loggerhead sea turtles

Mitogen[a]	*n*	Rs	*P*
PHA5	126	0.152	0.090
ConA 20	185	−0.131	0.076
LPS10	177	0.042	0.580
PDB 0.2	181	0.002	0.983

n: Sample size.
[a] Numbers next to mitogen names represent the final concentration (μg/ml) of mitogen in the culture wells.

4. Discussion

As lymphocyte proliferation, to our knowledge, had never been performed in loggerhead sea turtles, it was first important to optimize culture conditions for the assay. Optimization of this assay is critical in each new species to be examined, because individual species often require slight modifications of the assay, especially in mitogen concentration and incubation time. Normal physiological temperature ranges are also important to consider, especially for poikilotherms. These results demonstrate that loggerhead lymphocytes are indeed responsive to mitogen stimulation. The average SI using the ^{3}H-thymidine assay was 270 for PHA, 31 for ConA, 2.8 for LPS, and 2.5 for PDB (Fig. 6). The loggerhead responses to these mitogens were somewhat similar to responses

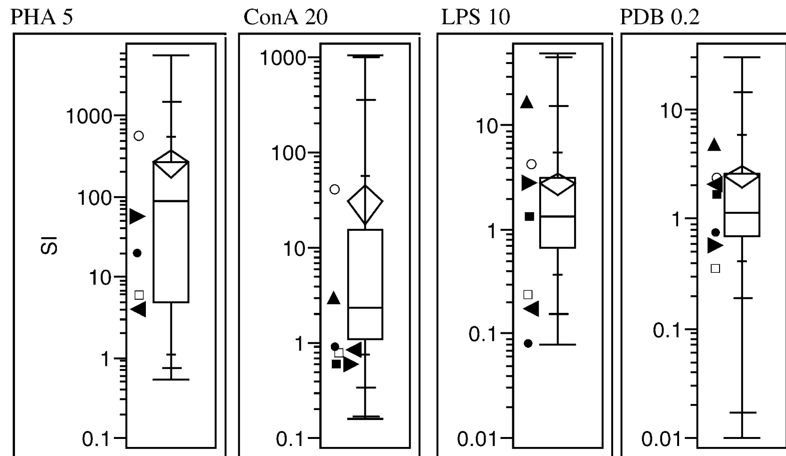

Fig. 6. Quantile box plots of mitogen-induced proliferation of lymphocytes obtained from loggerhead sea turtles captured in offshore waters of SC, GA, and northern FL in the summers of 2001–2003. Numbers next to mitogen names represent the final concentrations (μg/ml) of mitogen in the culture wells. SI: stimulation index (cpm from stimulated wells/cpm from unstimulated wells). The lines intersecting the top whisker, from the top down, are the maximum, the 99.5, 97.5, and 90% quantiles. The top of the box represents the 75% quantile, the line inside the box is the median, and the bottom of the box is the 25% quantile. The center of the diamond is the mean. The lines intersecting the bottom whisker, from the top down, are the 10, 2.5, 0.5% quantiles, and the minimum. Sample sizes are 168 for PHA, 230 for ConA, 223 for LPS, and 227 for PDB. The four turtles with the lowest body condition, assumed to be emaciated, are shown as (□) (turtle ID CC4003 with body condition index of 12.4), (●) (turtle ID CC0140 with body condition index of 11.0), (▲) (turtle ID CC6037 with body condition index of 12.2), and (⊞) (turtle ID CC6035 with body condition index of 12.9). The three turtles with visually sunken plastrons are shown as (○) (turtle ID CC2319 with body condition index of 14.1), (▶) (turtle ID CC2320 with body condition index of 15.3), and (▶) (turtle ID CC2364 with body condition index of 15.2).

previously reported for green turtles (Cray et al., 2001; Work et al., 2001). Of the T-cell mitogens, PHA produced a stronger response than ConA, whereas LPS and PDB resulted in similar B-cell responses. Although ConA produced an average SI of 31 ($n = 230$), it should be noted that ConA stimulated a strong proliferation response in some turtles and not in others. This large inter-individual variation was also noted by another laboratory that was optimizing conditions of the LP assay in loggerhead turtles during the same time frame in 2001 (K. Burnett, Per. Com.). The cause of this, however, is unknown and requires further investigation.

Direct comparison of the stimulation indices measured from the two methods showed that the ^{3}H-thymidine assay resulted in significantly higher SI than the MTT assay. A similar finding can be observed when comparing previously reported LP values for green sea turtles (Lutz et al., 2001; Cray et al., 2001; Work et al., 2001). These results were not totally unexpected. Based on studies with birds and mammals, the ^{3}H-thymidine assay typically produces higher SI than the MTT assay (Reubel and Bauerfeind, 1989; Chen et al., 1990; Bounous et al., 1992; Iwata and Inoue, 1993). One possible explanation of this difference may be the analytical method of measuring absorbance in the MTT assay. Spectrophotometers exhibit limited ranges over which they can accurately quantify absorbance. Values near zero are difficult to measure, and most instruments become non-linear (failing to follow the Beer-Lambert law) at approximately two absorbance units. Because unstimulated cells typically produce an absorbance of approximately 0.02–0.2, the theoretical maximum SI using MTT could range from 10 to 100. On the other hand, measuring radioactive uptake in the ^{3}H-thymidine assay is not physically limited to such a small range, so it can produce a higher SI. This analytical difference, however, cannot fully explain the SI of less than two which is often reported with the MTT assay (Chen et al., 1990; Bounous et al., 1992; Iwata and Inoue, 1993; Lutz et al., 2001). Iwata and Inoue (1993) suggested that a low SI from the MTT assay is due to relatively higher absorbance values for unstimulated cells, because this assay measures viability of not only actively dividing cells but also those that are resting.

Evidence for this was reported in unstimulated human lymphocytes by Chen and co-workers (1990). Regardless of the cause, the ^{3}H-thymidine method produces higher SI values and is, therefore, likely to demonstrate greater sensitivity by detecting finer scale changes than the MTT assay.

The lack of correlation between the MTT assay and the ^{3}H-thymidine assay supports the conclusion that the ^{3}H-thymidine assay is more appropriate for studies with loggerhead sea turtle PBLs. Many studies have reported good agreement between the two methods, including studies with chicken splenocytes (Bounous et al., 1992), swine PBLs (Iwata and Inoue, 1993), mice splenocytes (Mosmann, 1983), canine PBLs (Wagner et al., 1999), and bovine lymphocytes isolated from the blood, spleen and lymph nodes (Zolnai et al., 1998). On the other hand, disagreement between the two methods has been shown in several different studies. Recent work on the LP responses of spotted wolffish (*Anarhichas minor* Olafsen) PBLs demonstrated that the values obtained from the MTT assay did not agree with values obtained using the ^{3}H-thymidine assay (Espelid et al., 2003). A second study found that the MTT assay was relatively insensitive at detecting mitogen stimulation of swine PBLs compared with the ^{3}H-thymidine method (Reubel and Bauerfeind, 1989). Moreover, a third study examining LP using PBLs of mice, rats, and humans found no correlation between the SI obtained from the MTT assay and the ^{3}H-thymidine method (Chen et al., 1990). These three studies suggest that the non-radioactive MTT technique may not be the best choice for all species or all sample types, especially blood cells since each of the studies showing disagreement between methods used PBLs. The direct comparison in the current study between the two techniques using paired loggerhead turtle samples suggests that the ^{3}H-thymidine method is more appropriate for measuring the LP response in this species.

Various studies assessing mitogen-induced LP in loggerhead sea turtles have used either lithium (Peden-Adams et al., 2002) or sodium heparin (Keller et al., 2002) for blood collection. Because it is important to be able to compare results between studies it was critical to determine if heparin type influences the results of the LP response. As shown in this study there were no significant differences between results from paired samples collected in either lithium or sodium heparin, suggesting that heparin type does not influence the results of this assay. Therefore, heparin type should not be a confounding factor when comparing data from different studies evaluating LP in loggerhead turtles.

Gender differences are often seen in the immune response of various species, and steroid hormone concentrations are thought to play a key role in these gender differences (Schuurs and Verhuel, 1990; Zapata et al., 1992; Klein, 2000). Unexpectedly, gender did not significantly influence loggerhead LP. The PHA and LPS responses were higher in males than females, but this response was not statistically significant. To further evaluate the effect of hormones on the LP response, correlations between testosterone concentrations and LP were examined. Only one correlation was found to be statistically significant, but the association was weak ($Rs = -0.308$). This correlation suggested that juvenile females with lower plasma testosterone concentrations had stronger PDB responses. The lack of correlation in males is more notable, because the range of their testosterone concentrations (305–3555 pg/ml) is much larger than that for juvenile females (7.44–199.7 pg/ml). The lack of correlation in males was unexpected, because previous studies on seasonal changes of the reptilian immune system have shown that immune responses decrease when testosterone concentrations are high or when it is given by injection (Leceta and Zapata, 1985; Saad and Khalek, 1990; Saad et al., 1991). The results of the current study suggest that physiological concentrations of testosterone in the summer months do not greatly influence the LP response of loggerhead sea turtles; therefore, males respond similarly to females.

The body condition of the loggerhead turtles sampled for this study did not correlate with their LP responses. This result was also unexpected because Borysenko and Lewis (1979) had previously demonstrated that intentional malnourishment of snapping turtles resulted in signs of immunosuppression. The body condition index employed for this study was expected to reliably predict nutritional status in loggerhead sea turtles. Bjorndal et al. (2000) showed that this condition index for green sea turtles was sensitive to changes in both the growth rates of turtles and the population density. They demonstrated that during times when more turtles were feeding on a

limited amount of resources, turtle growth rates slowed and body condition decreased. Body condition indices for loggerhead turtles are not available in the literature, so a comparison could not be made between our observations and previous studies. However, body condition indices calculated in a separate study by this laboratory on emaciated loggerhead turtles ranged from 10.0 to 12.8 (unpublished data). The wide range of body condition (11.0–22.5) observed in the current study suggests that indeed some emaciated turtles were included. In fact, four of the turtles measured for both body condition and LP responses exhibited body condition indices below 12.9 and were assumed to be emaciated. Observations of sunken plastrons, a common sign of emaciation, were noted for an additional three turtles. The body condition indices of these latter turtles ranged from 14.1 to 15.3. When comparing the LP responses of these turtles to the range, median, and mean of all turtles measured for LP (Fig. 6), these turtles did not fall within the extreme low end of the LP responses. Thus, the lack of correlation suggests that nutritional status alone, as measured by this body condition index or visual observation, in free-ranging loggerhead turtles does not greatly influence this immune response. It is possible that this index developed in green sea turtles does not accurately reflect nutritional status of loggerhead turtles. Further studies may be needed to determine the appropriateness of this index for this species or other turtle species, or a larger sample size of emaciated turtles may be needed to statistically determine a relationship between body condition and LP responses.

5. Conclusion

This study developed and optimized the mitogen-induced LP assay in loggerhead sea turtles. Results indicate that loggerhead sea turtle PBLs are responsive to mitogens and that the ^{3}H-thymidine method may be more appropriate than the non-radioactive MTT method for measuring LP responses in loggerhead turtle PBLs. T- and B-cell proliferation was not greatly influenced by gender, testosterone concentrations, or body condition in loggerhead turtles. These results provide the framework for future research examining immunological function in loggerhead sea turtles compromised by disease and/or contaminants.

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