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RESEARCH PAPER

Intestinal B cells in the red-eared slider turtle, *Trachemys scripta*: Anatomical distribution and implications for ecological interactions with pathogenic microbes

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Abstract

Disease is a significant threat in the global decline of reptile species. Many aquatic reptiles live in habitats with high levels of opportunistic microbial pathogens, yet little is known about their immune system. Gut-associated lymphoid tissue is vital for protection against ingested pathogens and maintenance of normal gut microbiota. In mammals, gut mucosal immunity is well-characterized and mucosal surfaces are coated in protective antibodies. However, reptiles lack lymph nodes and Peyer's patches, which are the major sites of mammalian B cell responses. The presence or distribution of mucosal B cells in reptiles is unknown. In this study, we first set out to determine if B cells could be detected in intestinal tissues of red-eared slider turtles, *Trachemys scripta*. Using whole-mount immunochemistry and a primary antibody to turtle antibody light chains, we identified widely distributed B cell aggregates within the small intestine of hatchling turtles. These aggregates appeared similar to isolated lymphoid follicles (ILFs) in mammals and the frequency was much higher in distal intestinal sections than in proximal sections. To determine if these structures were inducible in the presence of microbes, we introduced an enteric *Salmonella* species through oral gavage. Analysis of intestinal tissues revealed that hatchlings exposed to *Salmonella* exhibited significantly more of these aggregates when compared with those that did not receive bacteria. These studies provide the first evidence for B cell-containing ILF-like structures in reptiles and provide novel information about gut immunity in nonmammalian vertebrates that could have important implications for ecological interactions with pathogens.

KEYWORDS
gut immunity, intestine, isolated lymphoid follicles, mucosal B cells, reptilian immunity, turtle

1 | INTRODUCTION

Disease is considered a significant threat in the global decline of reptile and amphibian species (Gibbons et al., 2000). For example, chytridiomycosis in amphibians (Van Rooij, Martel, Haesebrouck, & Pasmans, 2015) and snake fungal disease (Allender, Baker, Britton, & Kent, 2018) are significant conservation concerns in many populations. In recent years, the field of ecoimmunology has expanded our understanding of disease resistance in non-model species, yet much remains unknown. Aquatic turtles are long-lived ectothermic vertebrates that offer an excellent opportunity for studies on comparative animal physiology. Many species of turtles inhabit

wetlands and other habitats with high levels of potential opportunistic pathogens, increasing habitat fragmentation and pollution, and are negatively affected by global climate change (Hamilton et al., 2018; Silbernagel, Clifford, Bettaso, Worth, & Foley, 2013). Given that chelonians are among the most imperiled vertebrate groups (Rhodin et al., 2018), it is critical to understand their life history evolution. To do so, we must have a better grasp of how their immune system functions and their ability to respond to infection, both key components to fitness and survival.

Reptiles have both cellular and humoral immune compartments (Zimmerman, Vogel, & Bowden, 2010). They possess primary lymphoid tissues such as bone marrow and thymus (Borysenko & Cooper, 1972; Saad & Zapata, 1992; Zimmerman et al., 2010), but they lack certain secondary lymphoid tissues such as lymph nodes and Peyer's patches (PP), which are vital to mammalian immunity (Solas & Zapata, 1980; Zapata & Solas, 1979). Most previous studies on reptile immune function have focused on systemic immune responses, leaving important knowledge gaps in other aspects of their immune responses, such as local immune responses in mucosal tissues. Production of IgA by mammalian B cells is essential to prevent infection at body surfaces but reptile species differ genetically in the antibody isotypes they can produce (reviewed in Zimmerman et al., 2010). While IgA-like genes have been discovered in some non-chelonian reptiles (Sun, Wei, Li, & Zhao, 2012), turtles typically produce IgM, IgD, and IgY antibodies (Li et al., 2012; Pettinello & Dooley, 2014). Given that they may live in pathogen-rich environments, it is expected that turtles will have robust mucosal immunity, but virtually nothing is known about their mucosal immune response.

Gastrointestinal (GI) tissues are essential for nutrient absorption in all vertebrates but also serve as excellent areas for microbial colonization and invasion (Kato, Kawamoto, Maruya, & Fagarasan, 2014; Stevens & Hume, 1995). Therefore, the immune system must constantly patrol these tissues to prevent otherwise benign interactions from becoming pathogenic. Management of microbial colonization occurs through lymphoid tissues associated with the digestive system such as the tonsils, appendix, mesenteric lymph nodes, and PP. These structures house innate and adaptive immune cells that survey the GI tract and are commonly termed gut-associated lymphoid tissues (GALT; Brandtzaeg, Kiyono, Pabst, & Russell, 2008; Hamada et al., 2002; Stevens & Hume, 1995). In particular, PP are a major site for B cell responses in mammals and the primary site of IgA production in the small intestine (Jung, Hugot, & Barreau, 2010). Here, B and T cells interact in germinal centers to produce high affinity, isotype-switched antibodies. Given that turtles lack IgA and PPs, it is unclear how their gut microbiota is managed.

In addition to PPs and mesenteric lymph nodes in mammals, isolated lymphoid follicles (ILFs) contained within the digestive tract have recently gained attention due to their unique inducible nature and protective qualities (Kiss et al., 2011; Lee et al., 2012). ILFs have been found in mammals such as humans (Moghaddami, Cummins, & Mayrhofer, 1998), rabbits (Keren, Holt, Collins, Gemski, & Formal, 1978), mice (Hamada et al., 2002), and Guinea pigs (Rosner & Keren, 1984), and they are structurally distinct from the "traditional" GALT tissues such as PPs or mesenteric lymph nodes. ILFs are smaller and less obvious than PPs or mesenteric lymph nodes in the gut but are more numerous (Hamada et al., 2002; Keren et al., 1978). They appear earlier in development than PPs and are primarily composed of IgA producing B cells (Hamada et al., 2002). Unlike mesenteric lymph nodes and PPs, the distribution of ILFs can be influenced by changes in the surrounding microbial community to maintain homeostasis (Brandtzaeg et al., 2008; Knoop & Newberry, 2012).

GALT in reptiles has not been extensively characterized, with much of the literature consisting of descriptive studies with simple histological stains that are not able to identify specific cell populations. From these early studies almost 40 years ago, it is reported that there are possible lymphoid structures in the intestines of the common snapping turtle (*Chelydra serpentina*), with researchers noting diffuse aggregates composed of small mononuclear cells in the submucosa (Borysenko & Cooper, 1972). In a later comparative study, similar lymphoid structures were reported in close association with the lamina propria throughout the small and large intestine and in the cloaca of the caspian turtle (*Mauremys caspica*; Zapata & Solas, 1979). Similar structures were reported in the viperine water snake (*Natrix maura*), the Algerian sand lizard (*Psammodromus algirus*), and in the ocellated skink (*Chalcides occelatus*; Zapata & Solas, 1979). Thus, there is a possibility that reptiles could rely on small immune aggregates like the ILFs of mammals.

We hypothesized that due to lack of traditional GALT, mesenteric lymph nodes, and PPs, turtles will rely heavily on B cell lymphoid aggregates (e.g., ILFs) in their intestines to regulate gut immunity. Like mammals, we predicted that the introduction of microbes would increase the presence of these structures in the small intestine. To test our hypothesis, we used *Trachemys scripta* hatchlings to see if we could identify populations of B cell aggregates within their small intestines. First, the histological examination found clusters of mononuclear cells in the lamina propria of the intestines of these young animals, consistent with previous research in adults. Next, using immunohistochemistry with a turtle light chain-specific monoclonal antibody (mAb), we were able to successfully identify numerous small lymphoid aggregates that were composed of B cells. We then tested whether these aggregations had the ability to respond to changes within the gut microenvironment by introducing *Salmonella* to a subset of hatchlings. Our findings provide important novel information about mucosal defense in reptiles.

2 | MATERIALS AND METHODS

2.1 | Egg and hatchling care

All animals and eggs involved in the studies below were obtained from Banner Marsh State Fish and Wildlife Area in Canton, IL with approval by the Illinois Department of Natural Resources and the Illinois State University Institutional Animal Care and Use Committee. Females in our study population produce two clutches during the course of a single nesting season. Early season clutches are laid toward the end of May–early June, while late season clutches are

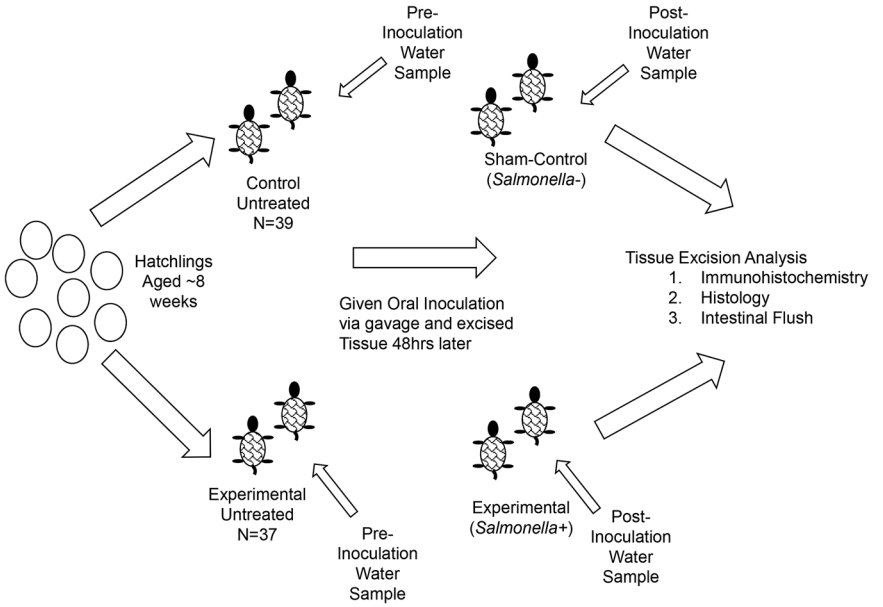

FIGURE 1 Experimental design. Eggs from multiple female clutches were collected and randomly assigned to sham or experimental groups. Hatchling were aged to 8 weeks before oral gavage. Water samples were taken pretreatment and after 48 hr. Hatchlings were euthanized and tissues prepared

laid near the end of June. Both season and clutch were considered in data analysis. Eggs were collected from freshly laid nests, or from gravid females that were collected from baited traps. Gravid females were transported to the laboratory and induced to oviposit by oxytocin (Agri-Laboratories) injection (Ewert & Legler, 1978). Eggs were incubated in moist vermiculite (approximately −150 kPa) until hatching. Once hatched, hatchlings were moved to individual containers and held for 8–10 weeks before the experimental treatment. Hatchlings were then randomly assigned to either the sham (*Salmonella*−) group ($N = 37$) or the exposed (*Salmonella*+) group ($N = 39$; Figure 1).

2.2 | *Salmonella* exposure

Salmonella enteritidis (ATCC 13076) was grown to an optical density (OD) of 0.3 (≈5×10^5 cells/ml) in Luria-Bertani (LB) broth the night before inoculations. One milliliter of these cells was concentrated via centrifugation, then resuspended in 20 µl of LB broth on the day of the inoculations. A 1 ml feeding tube containing 20 µl of *Salmonella* in LB broth for the experimental group or 20 µl sterilized LB broth for the sham group was administered to hatchlings via oral gavage.

To determine if *Salmonella* was present naturally in individuals before our deliberate exposure, water samples were collected from individual containers immediately before inoculation. Cups were then cleaned with a 10% bleach solution, rinsed thoroughly, and the oral inoculations were administered. Hatchlings were held for 48 hr to allow for colonization to take place. Water samples were taken after 48 hr, and hatchlings were euthanized by injection of pentobarbital sodium solution (>60 mg/kg). *Salmonella* presence was determined by plating water samples on Difco™ XLT4 (Thermo Fisher Scientific) selective agar plates, which were examined 24–28 hr later for the presence of black colonies.

2.3 | Small intestine histology

Sections of hatchling small intestine were collected and fixed in a 4% paraformaldehyde solution for 24 hr, then stored in a sucrose solution. Intestinal sections were then embedded in a paraffin wax cassette and sliced into 7 µm sections using a microtome. Sections were then stained using hematoxylin (Ricca Chemical) and eosin (Sigma Aldrich; Fletcher & Wibbels, 2014). Sections were photographed using a Leica DMRBE microscope. Histological sections were analyzed using the image processing software suite Fiji ImageJ v2.00 (Schindelin et al., 2012). Images were modified for correct white balancing using a macro written by Vytas Bindokas; Oct 2006, University of Chicago. Modified by Patrice Mascalchi.

2.4 | Whole-mount immunohistochemistry

To determine if B cells were present, we modified a whole mount immunohistochemistry technique (McDonald & Newberry, 2007), using a monoclonal antibody specific for turtle light chains (HL673; Herbst & Klein, 1995). We have previously found this mAb reacts with light chains from *T. scripta* in ELISA and flow cytometry (Zimmerman, Bowden, & Vogel, 2013; Zimmerman, Vogel, Edwards, & Bowden, 2010). After being flushed with phosphate-buffered saline (PBS) to remove any intestinal contents, intestinal tissue was prepared for immunohistochemistry. Two 1–2 cm sections were collected from each hatchling; one section was excised proximal to the yolk sac, which was located ~8 cm away from the stomach and the other section was excised distal to the yolk sac. Intestinal sections were cut longitudinally and mounted on Sylgard 184 Silicone epoxy (Dow Corning Corporation) in six-well tissue culture dishes lumen side up. Tissues were kept hydrated with room temperature 1× PBS, then were washed three times for 5 min each while shaking at 600 rpm in warm (37°C) Hank's balanced salt solution containing

0.1 M ethylenediaminetetraacetic acid (HBSS-EDTA) to remove the surface epithelial layer.

The intestinal sections were then washed with HBSS-EDTA using a 30cc syringe with a 23-gauge needle to ensure complete removal of the epithelial cell layer. Sections were then shaken for 10 min in cold (chilled on ice) 1× PBS and fixed with a 4% paraformaldehyde solution in 1× PBS for 1 hr. Following fixation, tissues were treated with 1% hydrogen peroxide in 1× PBS for 15 min to inactivate any endogenous peroxidases. Intestines were then blocked overnight in a 50 mM Tris buffer (pH 7.2) supplemented with 150 mM sodium chloride, 0.6% Triton-X 100, 0.1% bovine serum albumin (BSA), and 0.01% normal rat serum with shaking at 4°C. Following incubation, intestines were incubated overnight with a 1:500 dilution of antiturtle light chain mAb (HL673 mAb purchased from University of Florida Hybridoma Facility; Herbst & Klein, 1995) conjugated to biotin in the Tris buffer solution. The following day, intestines were washed three times for 10 min each with 1% BSA–1× PBS and incubated with streptavidin-horseradish peroxidase (SA-HRP; BD Biosciences) diluted 1:1,000 for 1 hr. After three washes, intestines were treated with diaminobenzidine peroxidase substrate (Metal Enhanced DAB Substrate Kit, Thermo Fisher Scientific) for 15 min. Finally, the intestinal sections were washed twice with dH_2O to stop any reactions and returned to 1× PBS. Images were taken at (×2.5) magnification using a Leica camera attached to a Leica dissecting scope.

2.5 | Spot proportion determination

Intestinal images were analyzed using the image processing software suite Fiji ImageJ v2.00 (Schindelin et al., 2012). Images were edited for white balancing using the aforementioned method in ImageJ. A grid pattern was placed on the images captured at ×2.5 magnification on the dissecting scope. All squares within grids had a surface area of 0.1 mm^2. Grid squares containing small intestine were counted for each image, and squares containing stained spots developed through immunohistochemistry to identify light chains associated with turtle B chains were also counted. Counts were done by two individuals, one of whom was blind to the treatments, and averaged. These values were used to calculate the proportion of imaged intestinal tissue exhibiting positive B cell staining.

2.6 | Statistical analysis

All statistical analyses were performed in R statistical software v3.3.3 (R Development Core Team, 2016) and the *lme4* package (Bates, Mächler, Bolker, & Walker, 2014). A generalized mixed model with binomial distribution was performed on the presence or absence of *Salmonella* in water samples collected both before and after treatment, and additionally on the proportion of small intestine grid areas with positive B-cell staining. For *Salmonella* presence in water, a model was fitted with *Salmonella* treatment as a fixed effect, and clutch as a random effect. The presence or absence of B cell staining within small intestine grids was analyzed with treatment, location (proximal/distal), and season (early/late) as fixed effects, with random effects of clutch origin and hatchling, to account for multiple measurements being taken from the same individual. Maximal models including possible interactions between fixed effects were simplified by sequentially eliminating nonsignificant terms through likelihood ratio tests, and best-fitting models were chosen based on the Akaike information criterion. For factor level comparisons, the package *lsmeans* (Lenth, 2016) was used to extract predicted probabilities for treatment levels from the best fitting models.

3 | RESULTS

3.1 | Intestinal histology reveals ILF-like structures in hatchling turtles

While early histological studies identified mononuclear cells presumed to be lymphocytes by morphology in adult reptilian gut tissues (Borysenko & Cooper, 1972; Solas & Zapata, 1980; Zapata & Solas, 1979), the distribution of specific B cells in gut tissue is unknown. First, we examined intestinal sections from hatchling turtles stained using hematoxylin and eosin to confirm we could see similar structures and that they were present in young animals. Intestinal histology slices showed aggregations of cells with lymphocyte-like cell morphology and placement in the lamina propria (Figure 2) consistent with previous reports in adult animals. There were no apparent afferent or efferent vessels, indicating that aggregates were formed locally, as opposed to a PP-like structure with drainage to a local lymph node. Slices with these structures were not continuous in all sections of the intestine and were easily distinguishable from a blood vessel (Figure 2a), which was observed to be continuous in all sections. Our initial observation supports that these structures are lymphoid aggregates, but this general staining method does not allow us to confirm what specific cell types are present.

3.2 | ILF-like structures contain B cells and are more abundant in the distal portion of the intestine

Using the modified whole mount immunohistochemistry technique, we found that no aggregate staining was observed in the absence of primary antibody (Figure 3a), while intestinal sections from untreated hatchling animals were found to contain B cell aggregations when incubated with the antiturtle light chain mAb (Figure 3b,c). The proportion of spots observed within the small intestine showed a highly significant difference by location, with distal sections containing a higher proportion of spots compared to the proximal sections (Figure 4; $\chi^2 = 162.97$, $df = 1$, $p < .001$).

3.3 | *Salmonella* exposure increases ILF-like aggregations

Hatchlings were randomly assigned to sham or experimental groups and preinoculation water samples tested. Both groups of hatchlings showed low natural *Salmonella* colonization as expected (Figure 5). There was no significant difference in the presence of *Salmonella* preinoculation among individuals randomly assigned to the two experimental groups

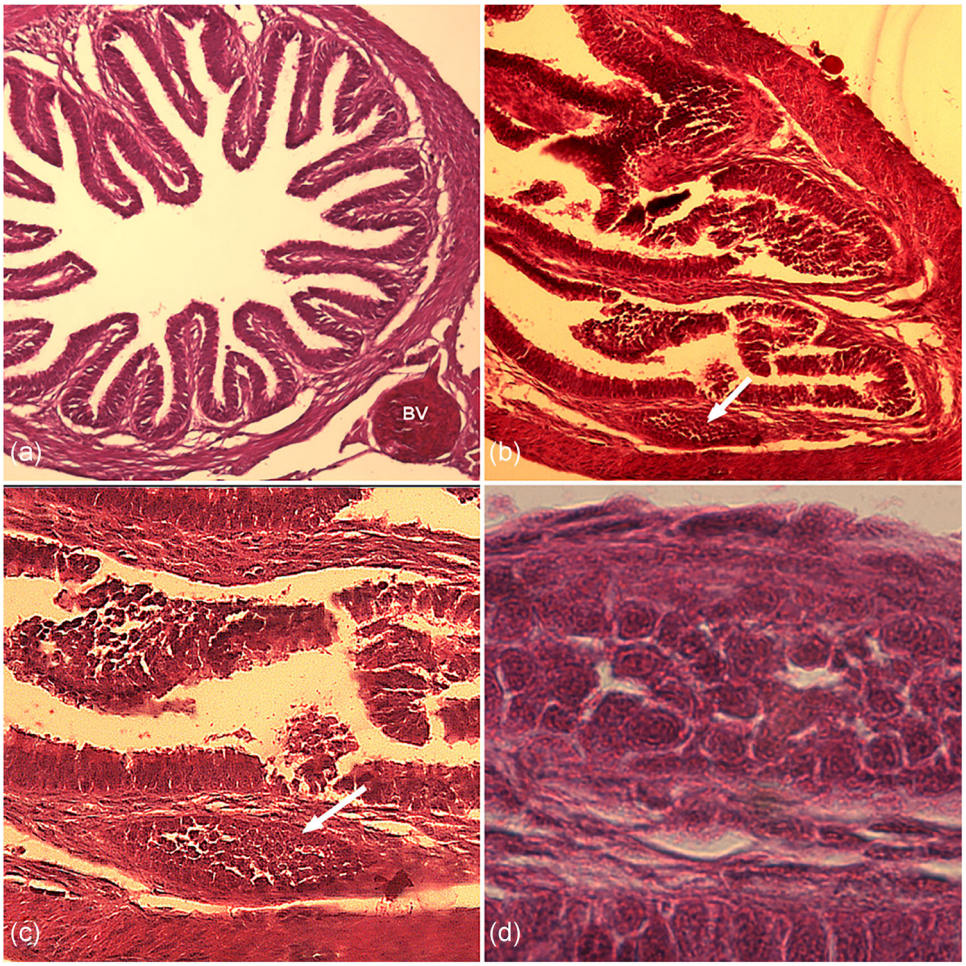

FIGURE 2 Aggregates of mononuclear cells are present in hatching intestinal tissue. Hatchling small intestines were sectioned (7 μM) and stained with H&E as described in Section 2. Representative images are shown. (a) Intestinal section with no lymphoid aggregation present; BV (×10). (b) Section with visible lymphoid aggregations, indicated by arrows (×20). (c) and (d) Same image as in (b) magnified to show the aggregates. (c) ×40 and (d) ×100. BV, blood vessel [Color figure can be viewed at wileyonlinelibrary.com]

($\chi^2 = 1.19$, $df = 1$, $p = .276$). However, 48 hr following inoculation, the number of individual water samples testing positive was significantly greater in the group that received the oral gavage containing *Salmonella* (Figure 5; $\chi^2 = 11.50$, $df = 1$, $p < .001$). Thus, it is likely that these animals were shedding bacteria in water samples or at least being exposed to the bacteria from their environment.

When we examined intestinal sections for the presence of spots developed through whole-mount immunohistochemistry, we observed a significant difference in the proportion of spots contained within the intestine between our sham and exposed treatment groups. Hatchlings inoculated with *Salmonella* had a higher proportion of spots when compared to those that were given the sham treatment (Figure 6; $\chi^2 = 5.75$, $df = 1$, $p = .017$). These findings indicate that exposure of hatchlings to *Salmonella* via oral gavage was successful, and also indicates that the observed B cell aggregations found in the small intestine are responsive to the presence of this microbe. Season had no significant effect on the proportion of spots contained within the intestine ($\chi^2 = 0.456$, $df = 1$, $p = .499$).

4 | DISCUSSION

Mucosal immunity and GALT structures in reptiles have received relatively little study compared with those of mammals. In our study, we sought to determine if and how B cells were distributed in the small intestine of the turtle, *T. scripta*. We hypothesized these cells play an important role in maintaining commensal microbiota and protection from pathogens. Given the lack of lymph nodes in reptiles, we predicted that the B cells would be found in numerous lymphoid follicle-like structures. We were able to identify lymphoid cell aggregations in the small intestine histologically. Using a monoclonal antibody specific for turtle antibody light chain proteins, we were able to clearly visualize small B cell clusters throughout the intestine. We also found the number and distribution of these structures could be influenced by location within the intestine and by exposure to bacteria. To the best of our knowledge, this is the first study that has specifically identified B cell aggregates within the intestine of a reptile.

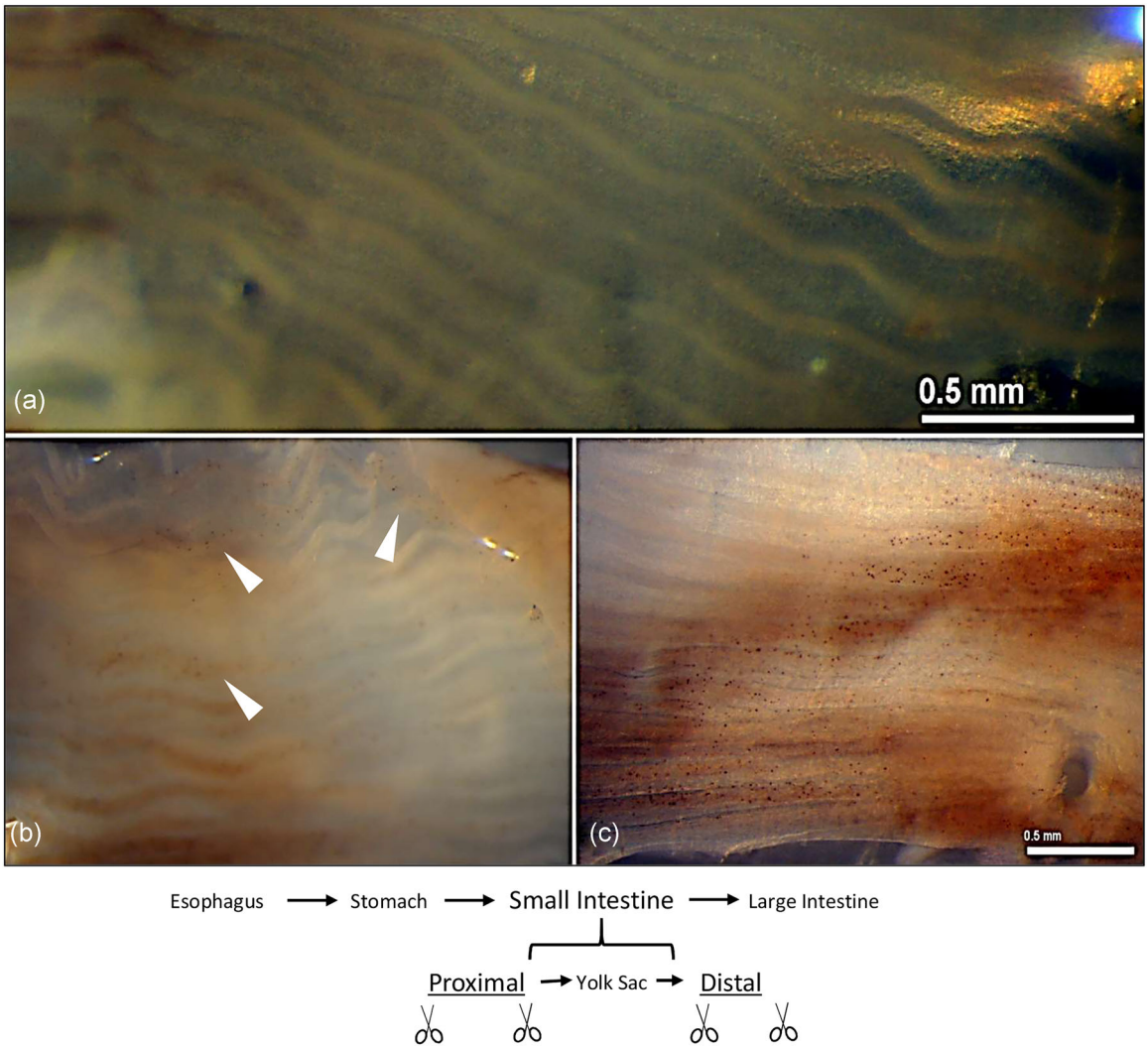

FIGURE 3 Clusters of B cells are present throughout hatchling intestines. Representative intestinal images are shown. (a) Negative control intestinal section that received no primary antibody. Proximal (b) and distal (c) intestinal sections stained with primary antibody to turtle light chains. Scale bars and magnifications are the same for images (b) and (c) [Color figure can be viewed at wileyonlinelibrary.com]

Many reptile species inhabit microbe-rich environments, such as wetlands, which are also experiencing an increase in the presence of potential pathogens due to global climate change and human needs for land and natural resources (Derne, Weinstein, & Lau, 2015). Recent studies have identified numerous pathogens associated with mortality events in turtles, including necrotizing bacterial infections, frog virus-3-like ranavirus, terrapene herpesvirus 1, adenovirus, *Mycoplasma*, *Salmonella*, and *Leptospira* (Adamovicz et al., 2018; McKenzie et al., 2019; Rockwell, Thompson, Maddox, & Mitchell, 2019). Many of these pathogens are spread by contact with mucosal surfaces; thus, an understanding of threats to turtles and conservation efforts may be enhanced with a more detailed understanding of mucosal immune protection. For example, in principle, oral vaccines might be designed to specifically stimulate mucosal immunity in designated populations of turtles during a disease outbreak. This approach is a cost-effective method to immunize wildlife and has been successfully used to prevent rabies in many species, swine fever in wild boars, tuberculosis in wild badgers, and plague in prairie dogs (Carter et al., 2018; Newton et al., 2019; Rossi et al., 2010; Tripp, Rocke, Runge, Abbott, & Miller, 2017).

It is important to note that this study was done in hatchling turtles, and our results indicate that hatchlings as young as 8 weeks have the potential to develop these B cell aggregations in the intestinal tract. These lab-raised individuals may have a lower overall microbial load and less B cell development than in adults. Thus, the future examination of these tissues in adults could provide further important information on the formation and distribution of ILF-like B cell aggregations.

B cell aggregates were more numerous in distal sections of hatchling intestines compared with the proximal sections. These findings are consistent with previous literature using nonspecific histological stains to examine potential GALT structures and lymphocytes in *M. caspica* , with more migrating lymphocytes and lymphoid tissue being observed as they progressed through the small intestine (Solas & Zapata, 1980; Zapata & Solas, 1979). Our findings are also consistent with the increased ILF presence in distal mouse intestines (Hamada et al., 2002; Lorenz, Chaplin,

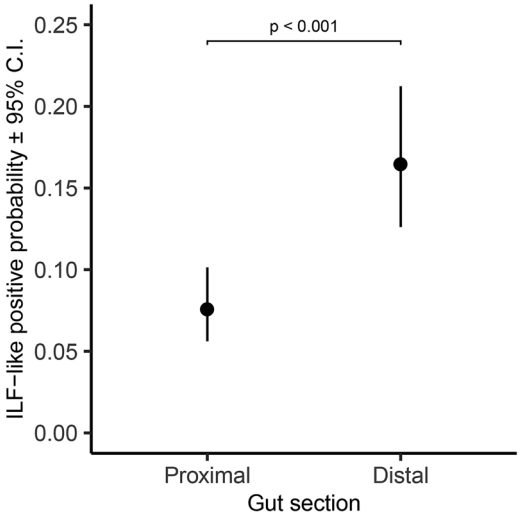

FIGURE 4 Frequency of B cell aggregates is increased in distal sections. Spot proportion was determined as described in Section 2. Points represent model-estimated probabilities. Distal sections ($n = 45$) and proximal sections ($n = 41$)

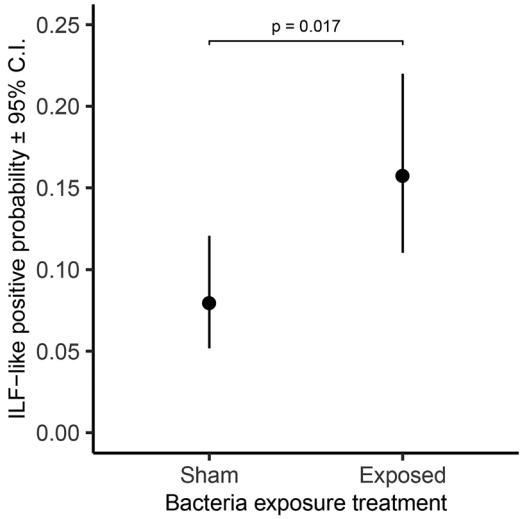

FIGURE 6 A higher proportion of spots were observed in hatchlings that were exposed to *Salmonella*. Whole mount immunohistochemistry of hatchling intestines was performed 48 hr after oral gavage. Images were analyzed as described. Points represent model-estimated probabilities. Sham-treatment group ($n = 38$), *Salmonella*-exposed group ($n = 48$)

McDonald, McDonough, & Newberry, 2003), suggesting that the distal portion of the small intestine is a particularly important site for ILF-like structures, likely due to the higher loads of bacteria found further in the digestive tract (Donaldson, Lee, & Mazmanian, 2016).

Based on reports in other species, we predicted aggregates would increase as the intestinal microbiota changed. Turtles are often carriers of *Salmonella* in the wild, however, healthy turtles are generally asymptomatic (Chiodini & Sundberg, 1981). We examined the influence of intentional oral gavage with *Salmonella* on the numbers and distribution of the aggregates. Our prediction was supported by a significant increase in aggregates in exposed versus sham individuals. Future experiments using a more pathogenic enteric bacterium or immune stimulant may cause a greater increase in ILF number. It has been shown in mammals that methods of pathogen recognition do not differ between commensal and pathogenic bacteria (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004), but methods for pathogen clearance have been shown to be different (Haller et al., 2000) and could alter the need for B cells in the intestine when more pathogenic bacteria are present. In reptiles, *Salmonella* is suggested to have evolved toward commensalism from a more pathogenic ancestor (Bäumler, Tsolis, Ficht, & Adams, 1998), which may only trigger limited B cell recruitment. Thus, exposure to a different, the more pathogenic microbe may change the aggregate distribution.

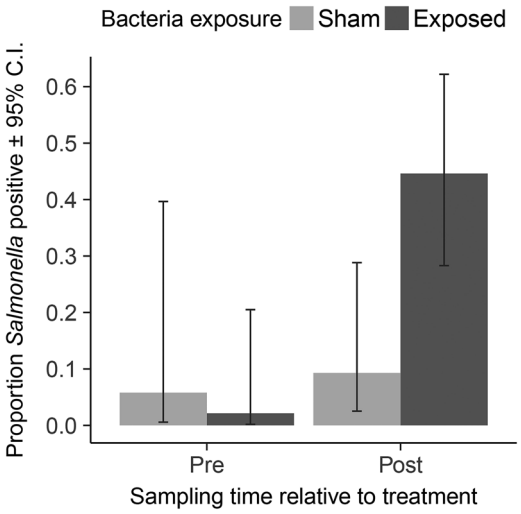

FIGURE 5 The number of individuals positive for *Salmonella* is low before treatment but increases in experimentally inoculated group. Hatchlings were randomly placed in sham (light gray) or exposure groups (dark gray) and water samples collected and plated on selective media as described. After 48 hr, a posttreatment water sample was also tested. Bars represent model-estimated proportions. Pretreatment Sham group ($n = 36$), pretreatment Salmonella-exposed group ($n = 38$), posttreatment Sham group ($n = 32$), posttreatment Salmonella-exposed group ($n = 38$)

We tested to see if any of our animals were carriers of *Salmonella* before our experimental exposure. We predicted natural carriage rates would be low due to being reared under laboratory conditions versus their normal soil habitat. Previous work by our laboratory and others did find that hatchling turtles could acquire *Salmonella* from ingesting contaminated eggshells during hatching (Holgersson, Nichols, Paitz, & Bowden, 2016), yet, the carrier state did not persist more than about 8 days post hatch (Pasmans, De Herdt, Dewulf, & Haesebrouck, 2002). Since our hatchlings were 8 weeks of age, we predicted a low incidence of *Salmonella* and indeed found low levels in preinoculation water samples. Postinoculation water sampling showed similarly low levels of *Salmonella* in the sham treatment, but a much higher presence in the exposed group. Ideally, water samples containing shed microbes should serve as a proxy for bacterial

colonization in the gut. However, during *Salmonella* inoculations, bacterial cells may have coated the mouth or head of the hatchling and been introduced to the water cup without actually colonizing the intestine. While this is not ideal, the environment experienced by the exposed treatment group would still be enriched for *Salmonella*. Continued environmental exposure and ingestion, not just colonization, would be predicted to have consequences for gut immunity.

Our laboratory has previously shown that B cells in *T. scripta* can secrete antibodies and perform phagocytosis (Zimmerman et al., 2010). It would be interesting to know if the B cells in ILF-like structures that we observe are antibody secreting, and, if so, what isotypes are produced in the absence of genes for IgA in turtles. Additionally, given the potential phagocytic functionality of B cells (Zimmerman, Vogel, Edwards et al., 2010), do these aggregates contain cells capable of phagocytosis, and what is their distribution? These are questions that we hope to address in future studies.

In conclusion, we have demonstrated the presence of inducible lymphoid cell aggregations in hatchling turtles. This not only increases our knowledge of mucosal immunity in reptiles but is relevant for our understanding of how turtles may cope with life in microbe-rich environments. The inducible nature of the aggregations suggests that turtles may be able to respond to the prevailing microbial environment, which will, in turn, dictate ecological interactions with potential pathogens and ultimately their individual health and fitness.

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