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Marine leech parasitism of sea turtles varies across host species, seasons, and the tumor disease fibropapillomatosis

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ABSTRACT: Fibropapillomatosis (FP) is a tumorous disease affecting all species of sea turtles and is associated with the pathogen chelonid alphaherpesvirus 5 (ChHV5). Hypothesized ChHV5 vectors include the marine leeches *Ozobranchus branchiatus* and *O. margoi*, but data on their associations with FP and ChHV5 are minimal. To establish relationships between leech parasitism, turtle hosts, and FP, we compared green and loggerhead turtles from the Indian River Lagoon (IRL), Florida, USA, in terms of (1) the presence or absence of ChHV5 within associated leeches, (2) the association between leech parasitism and host FP status, and (3) seasonal variation in leech presence. We identified 55 leeches collected from green turtles as *O. branchiatus* and 22 leeches collected from loggerhead turtles as *O. margoi*. Of 77 sequenced leeches, 10 *O. branchiatus* and 5 *O. margoi* were ChHV5 positive. ChHV5-positive *O. branchiatus* trended towards coming from FP-positive hosts. Using 12 yr of turtle capture data from the IRL, we found that leech parasitism was significantly correlated with FP and capture month in green turtles but not in loggerhead turtles. These results suggest that *O. branchiatus* and *O. margoi* may differ in their ability to transmit ChHV5 or to encounter and remain on FP-positive hosts. Alternatively, potential immunological differences between green and loggerhead turtles may explain the observed relationships. This study is the first to provide robust statistical evidence of an association between leeches and FP, as well as seasonal fluctuations in leech presence, in green turtles but not in loggerhead turtles.

KEY WORDS: *Ozobranchus* · Vector · ChHV5 · Indian River Lagoon · *Chelonia mydas* · *Caretta caretta*

1. INTRODUCTION

In recent decades, emerging infectious diseases (EIDs) of wildlife populations have increased dramatically (Jones et al. 2008), posing a threat to ecosystem health and biodiversity by causing population declines or extinctions (Daszak et al. 2000). These EIDs may be driven by several factors, including the global trade of host species by humans (Karesh et al. 2005), changes in host population density (Daszak et al. 2000, Hochachka & Dhondt 2000), and fluctuations in climate (Harvell et al. 2002). One such EID is the tumorous disease fibropapillomatosis (FP), which has emerged in threatened and endangered sea turtles (Lucké 1938, Smith & Coates 1938).

FP is a neoplastic disease affecting sea turtles and is characterized by the development of external and internal tumors (Lucké 1938, Smith & Coates 1938, Herbst 1994). These tumors are rarely directly fatal but may result in cases of mortality or decreased health if feeding, movement, or vision are significantly impaired (Herbst 1994, Adnyana et al. 1997). Since its description (Lucké 1938, Smith & Coates 1938), FP has been documented in all 7 sea turtle species (reviewed in Jones et al. 2016) and has become epizootic (Herbst 1994). This disease prima-

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rily affects juvenile sea turtles in neritic areas (Ene et al. 2005) and is a panzootic in green turtles *Chelonia mydas* (reviewed in Jones et al. 2016). Generally, among all sea turtle species, FP prevalence is highest in green turtles but varies geographically and temporally (Herbst 1994). While several locations have seen an increase in FP prevalence (Ehrhart et al. 2016, da Silva-Júnior et al. 2019, Shaver et al. 2019), others remain FP free or have experienced recent declines in prevalence (Baptistotte 2007, Chaloupka et al. 2009, Patrício et al. 2011). The epidemiology of FP is not well understood, though hypothesized factors of disease expression include environmental contaminants (Adnyana et at. 1997, Foley et al. 2005, Keller et al. 2014) and the turtle's life stage and geographic location (Herbst 1994, Ene et al. 2005, Work et al. 2020). The development of FP likely cannot be attributed to 1 cause but may instead be determined by a complex interplay of factors (Jones et al. 2016).

The epidemiology of FP suggests an infectious agent as the primary cause of tumor development. Herbst et al. (1995) used cell-free tumor extracts to cause FP tumor formation and thus concluded that FP is most likely horizontally transmitted by a virus, which was later identified as chelonid alphaherpesvirus 5 (ChHV5) (Quackenbush et al. 1998). ChHV5 is thought to be acquired by turtles once they recruit to neritic bays (Ene et al. 2005). The virus's association with tumor formation, as well as the host's response to ChHV5 infection, can vary across populations and may explain the variation in FP dynamics across these populations (Work et al. 2020). Hypotheses for how ChHV5 spreads include the presence of superspreaders (Work et al. 2015), transmission through the environment (Page-Karjian et al. 2015), and the presence of a vector organism (Lu et al. 2000, Greenblatt et al. 2004).

Vector organisms may be classified as biological or mechanical. Biological vectors are those that transmit a pathogen from one host to another while the pathogen replicates within the vector (Harwood & James 1979). Conversely, the pathogen does not replicate within a mechanical vector (Harwood & James 1979). Historically, a number of mechanical vectors have been suggested for FP and ChHV5, including cleaner fish (Lu et al. 2000), marine leeches (Nigrelli & Smith 1943, Greenblatt et al. 2004), and spirorchid trematodes which have since been rejected as potential vectors (Herbst et al. 1998). Greenblatt et al. (2004) investigated ChHV5 viral loads in amphipods, bladder parasites, barnacles, blood flukes, and marine leeches from the genus *Ozobranchus* and found that only larval and adult *Ozobranchus* species contained sufficient viral loads to be considered possible mechanical vectors.

The *Ozobranchus* genus contains 2 species of marine leeches that parasitize sea turtles: *Ozobranchus margoi* and *O. branchiatus* (Sawyer et al. 1975, Greenblatt et al. 2004). Both species are ectoparasites that feed on blood, attach to the soft tissue of sea turtles, and seem to exhibit strong host specificity (Bunkley-Williams et al. 2008). *O. margoi* primarily parasitizes loggerhead turtles *Caretta caretta* (Sawyer et al. 1975) but has been found on 3 other sea turtle species, including green turtles (reviewed in Bunkley-Williams et al. 2008). *O. branchiatus* mainly parasitizes green turtles (Sawyer et al. 1975) but has been found on 4 other sea turtle species, including loggerhead turtles (reviewed in Bunkley-Williams et al. 2008, McGowin et al. 2011). Leeches often cluster on FP tumors, potentially influencing FP development (Nigrelli & Smith 1943, Greenblatt et al. 2004) or possibly being attracted to FP tumors due to the high vascularization in tumors (Ehrhart 1991, Burkhalter & Norton 2019). However, studies on the association between marine leeches and FP or ChHV5 lack either large sample sizes or statistical support (Nigrelli & Smith 1943, Greenblatt et al. 2004, Bunkley-Williams et al. 2008, McGowin et al. 2011).

Here, we investigated differences between green and loggerhead turtles in terms of (1) the presence or absence of ChHV5 within associated leeches, (2) the association between *Ozobranchus* spp. parasitism and host FP status, and (3) seasonal variation in leech presence. Our sample sites consisted of 2 important neritic developmental habitats for juvenile turtles: the Indian River Lagoon (IRL), Florida, USA (Ehrhart et al. 2007), and the Trident Submarine Basin, Port Canaveral, Florida, USA (Ehrhart et al. 2016). FP prevalence has averaged around 50% among captured green turtles in the IRL since 1983 (Hirama & Ehrhart 2007, Borrowman 2008). The first reported case of FP in the Trident Submarine Basin occurred in 2005, and FP prevalence increased rapidly to 17.5% by 2015 (Ehrhart et al. 2016). FP does not impact annual apparent survival rates in these study sites (Borrowman 2008) nor overall population growth (Hirama & Ehrhart 2007). However, stranded turtles with FP are more likely to be emaciated than stranded turtles without FP in Florida, indicating a potential impact of FP on overall health (Foley et al. 2005). We quantified ChHV5 viral load in a cohort of leeches we sampled and sequenced in 2017 and 2018. We also analyzed recorded data on FP status, morphometric measurements, leech presence, and

season from over 2000 turtles captured in the IRL from 2006 to 2018. This study advances our understanding of the epidemiology of FP by assessing the relationship between *Ozobranchus* spp. parasitism, ChHV5 infections, and sea turtle disease.

2. MATERIALS AND METHODS

2.1. Sample collection

Data were collected in the IRL by the University of Central Florida (UCF) Marine Turtle Research Group (MTRG) twice a month from January 2006 to December 2018. Large-mesh tangle nets (0.5 km long) were set and monitored for 3 h per sampling day (Ehrhart et al. 2007). Captured turtles were transferred to a work-up boat, where data on turtle FP status, morphometric measurements, species, and leech presence were recorded. Larval and adult leech age classes were not distinguished, and leech eggs were reported separately. Approximately 2 to 5 ml of whole blood was collected from captured turtles from the dorsal cervical sinus and was frozen at −20°C until DNA was extracted, within 24 mo. Each turtle was tagged with 2 flipper tags and a passive integrated transponder tag, then released into the surrounding area. Leech individuals were collected opportunistically from juvenile green and loggerhead turtles, with and without FP, from January 2017 to December 2018 during bimonthly sampling days. Leeches were removed using tweezers that were disinfected with 70% isopropyl alcohol swabs before and after each sample collection. Leech specimens collected from the same host were stored at room temperature in 2 ml collection tubes in 70% ethanol.

During March 2017, leech samples were also collected opportunistically by the UCF MTRG during a 2 d semiannual sampling effort in the Trident Submarine Basin, Port Canaveral, FL, a popular foraging ground for juvenile sea turtles roughly 60 km north of the IRL site. Turtles were captured using large-hoop dip nets and large- and small-mesh tangle nets. Data and sample collection followed the procedures used during the IRL sampling efforts described above.

2.2. Genomic DNA extraction

We extracted genomic DNA (gDNA) from individual leeches using DNeasy Blood and Tissue Kits (Qiagen). All leeches were rinsed with deionized water to minimize contamination from the environment as well as to wash any viral contamination on their external mouthparts. If the leech was smaller than 3 mm^2, then the entire leech was used for DNA extraction. If the leech was larger than 3 mm^2, then the leech was cut along its midline to produce approximately 3 mm^2 of tissue for DNA extraction. Between individuals, scalpels were flame sterilized using 100% ethanol. We also extracted gDNA from 20 µl of nucleated blood from corresponding hosts using the Qiagen DNeasy Blood and Tissue Kit. For all DNA extractions, we used 50 µl of Buffer AE for the first eluate instead of the standard 200 µl to increase DNA concentration. We then used 200 µl of Buffer AE to generate a second, less concentrated eluate. All DNA extractions were stored at −20°C until later analyses, which occurred within 6 mo of extraction.

2.3. Cytochrome *c* oxidase I amplification and sequencing

Because visual identification of species can be challenging due to the leeches' small sizes and minor morphological differences (McGowin et al. 2011), we sequenced a DNA barcoding region to identify each leech sample to species. Specifically, we sequenced a 685 base pair fragment of the cytochrome *c* oxidase I (CO1) mitochondrial gene because it contains highly conserved regions where primers can be designed as well as variable regions that can distinguish species (Hebert et al. 2003). Using PCR, we amplified the CO1 gene fragment from leech gDNA. Due to the sensitivity of PCR amplification of the CO1 gene, we were able to use the more dilute DNA eluate of 200 µl of Buffer AE. We used the universal Folmer primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) for the forward primer and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) for the reverse primer (Folmer et al. 1994). Each PCR reaction consisted of 5.0 µl of 10× iTaq buffer (Bio-Rad), 1.0 µl of 10 mM dNTP mix, 1.0 µl of 10 µM forward primer, 1.0 µl of 10 µM reverse primer, 0.25 µl of iTaq DNA polymerase, 11.75 µl of molecular grade water, and 5.0 µl of the leech template DNA. We followed the PCR thermal regime described in McGowin et al. (2011), using a T100 Thermal Cycler (Bio-Rad). All PCR amplification products were run on a 2% agarose gel to confirm successful amplification. Samples that had clear bands of the expected size were cleaned using ExoSAP PCR Product Cleanup Reagent (Thermo Fisher Scientific) and

sent to Eurofins Genomics, where they were Sanger sequenced in both directions.

2.4. Species identification and phylogeny reconstruction

We cleaned and aligned resulting CO1 sequences in Geneious v.11.1.2 (Kearse et al. 2012). To assign each of our leech samples to species, we compared our recovered sequences to the DNA barcodes developed by McGowin et al. (2011) for *Ozobranchus margoi* and *O. branchiatus*. Entering our generated sequences into NCBI's BLAST, we identified all significant hits that were *Ozobranchus* spp., including *O. margoi*, *O. branchiatus*, and the freshwater species *O. jantseanus*. Among hundreds of nearly identical BLAST hits, we included 24 highly similar sequences (pairwise percent identity ranging from 79.9 to 100%; mean of 92.3%) in our phylogeny to place our sequences in the context of previous studies. To optimally root our phylogeny, we included GenBank sequences (Clark et al. 2016) from the freshwater leech *O. jantseanus* as a closely related outgroup and 2 arthropod taxa (*Leucophenga* sp., GenBank accession no. KP697105; *Uenoa lobata,* GenBank accession no. KY582937) as distant outgroups. We reconstructed a Bayesian phylogeny using these sequences from GenBank (Table S1 in the Supplement at www.int-res.com/articles/suppl/d143p001_supp.pdf) and our sequence data (Table S2). We used PartitionFinder v.2.1.1 (Lanfear et al. 2017) to determine models of evolution using both unlinked and linked branch length models and MrBayes v.3.2.7 (Huelsenbeck & Ronquist 2001) to reconstruct the phylogeny. The Bayesian analysis consisted of 2 runs of 4 independent Markov chain Monte Carlo (MCMC) chains run for a total of 5×10^6 generations each, with trees sampled every 100 generations. The first 100 000 iterations were discarded as burn-in. Results were visualized in Tracer v.1.7 (Rambaut et al. 2018) to confirm MCMC chain convergence and adequate sampling of the posterior distribution.

2.5. ChHV5 viral load quantification

We conducted quantitative PCR (qPCR) on all leech and turtle blood DNA using a modified version of the protocol developed by Page-Karjian et al. (2015) and validated across numerous tissue types by Lawrance et al. (2018) to quantify the number of ChHV5 gene copies (viral load) in each sample. Following Page-Karjian et al. (2015), we targeted the ChHV5 DNA polymerase region (UL30). Each well consisted of a 21.80 µl reaction containing 10 µl of SsoAdvanced Universal Probes Supermix (Bio-Rad), 0.80 µl of 10 µM forward primer (5′-AAC GCT TGC TTT TGG ACA AG-3′) (Integrated DNA Technologies [IDT]), 0.80 µl of 10 µM reverse primer (5′-CCA GCG GGT GTG AAT AAA AT-3′) (IDT), 2.00 µl of 1 µM ChHV5 pol probe (5′-6-FAM-TGG CCA TCA-ZEN-AGC TGA CGT GCA-3′) (IDT), and 8.20 µl of DNA template from the first, more concentrated eluate. We used the more concentrated DNA eluate to maximize the likelihood of amplification. Using a custom ChHV5 polymerase gBlocks gene fragment (IDT), we established standard curves for ChHV5 quantity ranging from 1.64×10^1 to 1.64×10^7 gene copies per reaction. DNA extracted from a confirmed ChHV5-positive FP tumor collected in the IRL was used as a positive control, and molecular grade water was used as a negative control. Reactions were run on a CFX96 Real-Time System (Bio-Rad) with the following reaction conditions: 10 min at 95°C, then 40 cycles of 30 s at 95°C and 1 min at 55°C. Any sample with a quantification cycle (Cq) value below cycle 38 was run a second time to validate positive results. Starting quantities (SQs) of ChHV5 gene copies were averaged from the first and second runs for samples that were positive across both runs. If a sample was negative on the second run, it was run a third time. If the Cq value was below cycle 38 on the third run, the sample was considered ChHV5 positive, the second run was considered a false negative, and the average SQ was taken from only the first and third runs. All results were analyzed using Bio-Rad CFX Manager software v.3.1. qPCR and subsequent analyses were conducted following MIQE guidelines (Bustin et al. 2009).

2.6. Statistical analyses

To test for the probability of finding a ChHV5-positive leech on a host with FP versus a host without FP, we ran a mixed effects logistic regression model with each individual host sampled in 2017 and 2018 as a random effect. We only used data from leeches collected from green turtles for this analysis due to the small loggerhead turtle dataset associated with our sequenced leeches. Additionally, we used data collected by the UCF MTRG from 1676 green turtle and 443 loggerhead turtle capture events in the IRL from January 2006 through December 2018 to test for significant associations

between *Ozobranchus* spp. parasitism and host species, host FP status, straight carapace length (SCL) of the host, body condition index (BCI) of the host, and seasonality. BCI was calculated as weight divided by SCL cubed, then multiplied by a factor of 1000 (Bjorndal et al. 2000). We used chi-square tests to determine whether FP or leech prevalence differed significantly between green and loggerhead turtles. We ran multiple generalized additive models (GAMs) to predict the presence or absence of larval and adult leeches for each host species, including combinations of the covariates FP presence or absence, SCL, BCI, and month (as a continuous variable) in each model, and then used corrected Akaike's information criterion scores to determine the most plausible model(s). We also ran separate GAMs to predict the presence or absence of leech eggs for green turtles, with month as a continuous variable, to determine whether seasonal variation in larval and adult leeches in this host species could potentially be explained by annual leech reproductive cycles. We considered $p < 0.05$ to be significant. We used R v.3.5.2 for all statistical analyses.

3. RESULTS

We extracted gDNA from 109 leech individuals. Of these, 76 amplified successfully using CO1 primers and were Sanger sequenced cleanly in both directions (Table S2 in the Supplement). Unsuccessful amplifications were likely due to low DNA concentration or the presence of PCR inhibitors (Lorenz 2012). One additional sample (GenBank accession no. MN481299) amplified successfully but only sequenced cleanly in the reverse direction. Based on these 77 CO1 sequences, 22 leeches were identified as *Ozobranchus margoi* (GenBank accession nos. MN481288–MN481309), and 55 were identified as *O. branchiatus* (GenBank accession nos. MN481310–MN481364). All sequenced *O. margoi* were collected from 2 loggerhead turtles, and all sequenced *O. branchiatus* were collected from 27 green turtles. In total, we generated sequence data from 19 *O. margoi* collected from a single FP-positive loggerhead turtle, 3 *O. margoi* collected from a single FP-negative loggerhead turtle, 32 *O. branchiatus* collected from 19 FP-positive green turtles, and 23 *O. branchiatus* collected from 8 FP-negative green turtles. All leeches were collected from the IRL, except the 3 *O. margoi* collected from a single FP-negative loggerhead turtle, which were collected from the Trident Submarine Basin. A complete record of host species and ID, host FP status, leech ChHV5 status, collection location, leech species, and GenBank accession number is provided for each leech specimen (Table S2).

Phylogenetic analysis recovered distinct species clades for *O. margoi*, *O. branchiatus*, and *O. jantseanus*, as well as distinct genetic lineages within each species (Fig. 1). *O. margoi* individuals separated into 2 lineages, one containing 24 *O. margoi* collected in Florida, USA, and Virginia, USA, and one containing a single individual collected in Taiwan. *O. branchiatus* individuals were distributed across 3 well-supported clades. One clade included only individuals collected from green turtles in Florida and contained all of our sequenced *O. branchiatus*. A second clade included *O. branchiatus* collected from Hawaii, Taiwan, Mexico, Hong Kong, and Brazil, and these individuals were collected from green turtles, olive ridley sea turtles *Lepidochelys olivacea*, or unspecified hosts. The third clade consisted of a single *O. branchiatus* collected from a loggerhead turtle in Florida. Among the *O. branchiatus* we collected and sequenced, 4 formed a distinct genetic lineage, while the remaining 51 individuals were nearly genetically identical. The 5 *O. jantseanus* collected from freshwater Reeves' turtles *Mauremys reevesii* in Japan or an unspecified host in China were distributed across 2 lineages.

Fifteen of the 77 sequenced leeches (19.5%) tested positive for ChHV5 using qPCR. Of these, 10 were *O. branchiatus* and 5 were *O. margoi* (Table 1). Of the 51 leeches collected from FP-positive hosts, 14 were ChHV5 positive (27.5%). In contrast, only 1 of 26 leeches from FP-negative hosts (3.8%) was ChHV5 positive (Table 1). The average viral load for the positive control FP tumor across multiple runs was 2302 ± 687 (mean ± SE) gene copies. One *O. branchiatus* individual had an average viral load of 21 928 ± 1125, while 1 *O. margoi* individual had a viral load of 40 211 ± 1598 (Table 2). Not including these 2 outliers, the average viral load among ChHV5-positive leeches was 647 ± 179 for all leeches, 619 ± 206 for *O. branchiatus*, and 794 ± 397 for *O. margoi*.

The mixed effects logistic regression to test for the probability of finding a ChHV5-positive leech on a green turtle host with FP versus a host without FP failed to converge. Consequently, we ran the same model without the random effect of individual host. From that model, ChHV5-positive leeches were more likely to be collected from FP-positive green turtles than from FP-negative green turtles ($Z = 2.153$, $p < 0.05$). However, because each host contributed a different number of leeches to our analyses, these statistical analyses provide limited in-

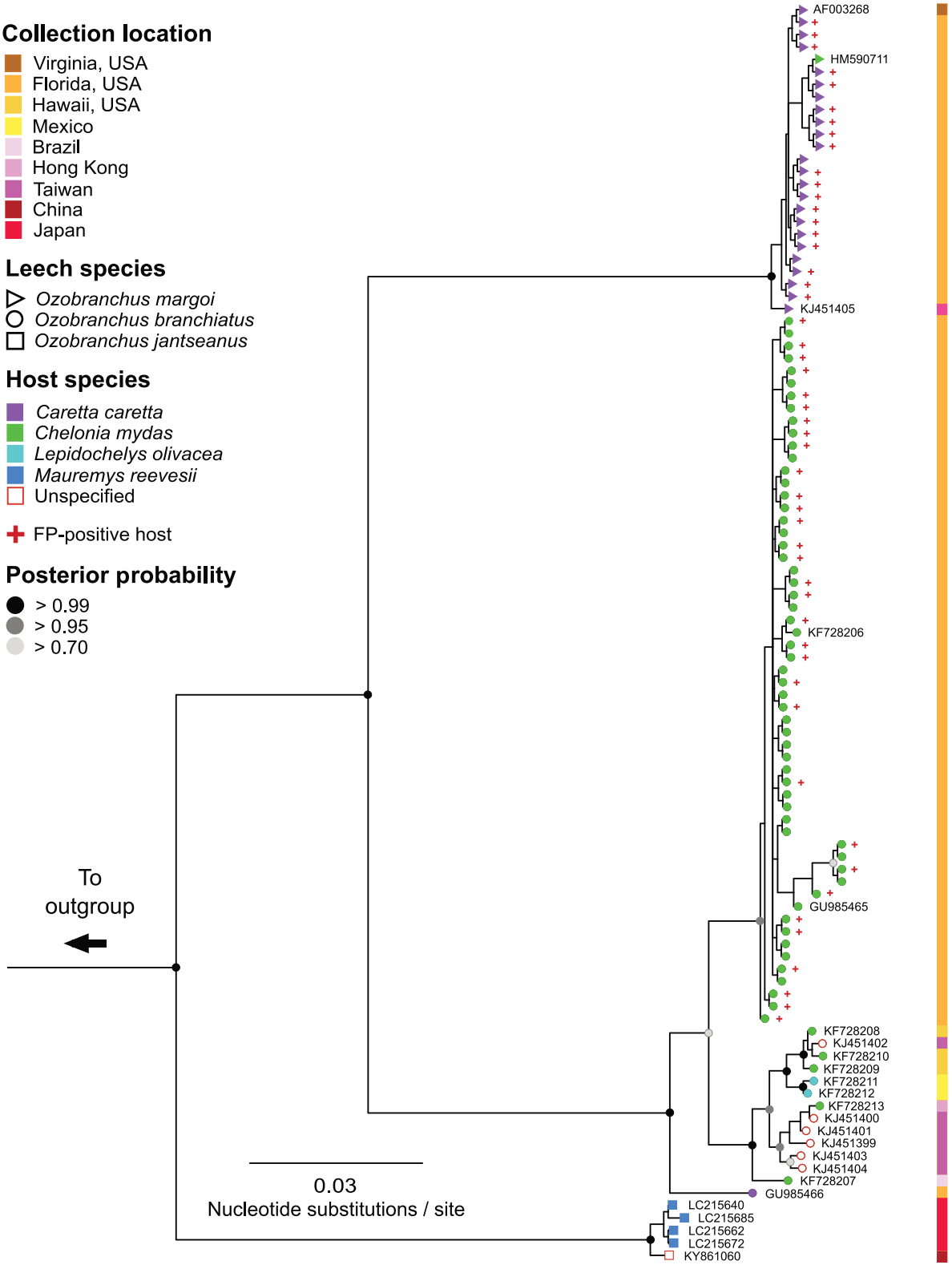

Fig. 1. Bayesian phylogeny reconstructed using MrBayes. Unless a GenBank accession number is provided, every sample was generated in this study. Host FP status is unknown for all GenBank-derived samples. *Leucophenga* sp. (KP697105, Huang & Chen 2016) and *Uenoa lobata* (KY582937, J. Xu & B. Wang unpubl. data) were used as outgroups

Table 1. Prevalence of chelonid alphaherpesvirus 5 (ChHV5) and average number of ChHV5 gene copies (viral load, ±SE) within leeches, grouped by leech species and by fibropapillomatosis (FP) status of the turtle host from which the leech was collected. Number of FP-positive hosts, total number of hosts, and host species are reported for each leech species

Leech sample	Host species (no. of FP-positive hosts/total hosts)	Leech ChHV5 prevalence	Viral load
Ozobranchus margoi	*Caretta caretta* (1/2)	5/22 (22.7%)	8463 ± 7943
O. branchiatus	*Chelonia mydas* (19/27)	10/55 (18.2%)	2807 ± 2132
From FP-positive host	Both turtle species	14/51 (27.5%)	5013 ± 3106
From FP-negative host	Both turtle species	1/26 (3.8%)	208

Table 2. Average number of chelonid alphaherpesvirus 5 (ChHV5) gene copies (viral load, ±SE) among ChHV5-positive *Ozobranchus margoi* and *O. branchiatus* and the fibropapillomatosis (FP) tumor positive control. Host ID, species, and FP status (+: positive; –: negative) are reported. Number of replicates indicates the number of times the sample was tested using quantitative PCR for ChHV5 viral load

GenBank accession no.	Species	Host ID	Host species	Host FP status	No. of replicates	Viral load
MN481293	*O. margoi*	53639	*Caretta caretta*	+	2	47 ± 17
MN481295	*O. margoi*	53639	*C. caretta*	+	2	1712 ± 531
MN481296	*O. margoi*	53639	*C. caretta*	+	2	129 ± 48
MN481308	*O. margoi*	53639	*C. caretta*	+	2	40211 ± 1598
MN481309	*O. margoi*	53639	*C. caretta*	+	2	216 ± 48
MN481311	*O. branchiatus*	52202	*Chelonia mydas*	+	2	154 ± 47
MN481315	*O. branchiatus*	52541	*C. mydas*	+	2	1356 ± 114
MN481322	*O. branchiatus*	52199	*C. mydas*	–	2	208 ± 31
MN481328	*O. branchiatus*	52197	*C. mydas*	+	2	1600 ± 47
MN481343	*O. branchiatus*	52540	*C. mydas*	+	2	21928 ± 1125
MN481349	*O. branchiatus*	52204	*C. mydas*	+	2	347 ± 17
MN481351	*O. branchiatus*	52204	*C. mydas*	+	2	589 ± 28
MN481353	*O. branchiatus*	52232	*C. mydas*	+	2	84 ± 9
MN481361	*O. branchiatus*	52240	*C. mydas*	+	2	1491 ± 274
MN481362	*O. branchiatus*	52204	*C. mydas*	+	2	319 ± 23
	FP tumor pos. control		*C. mydas*		5	2302 ± 823

sight. Furthermore, only 1 of the 15 ChHV5-positive leeches was collected from an FP-negative turtle; therefore, statistical tests investigating a correlation between leech viral load and host FP status were not feasible. None of the turtle whole blood samples tested positive for ChHV5. ChHV5 viral load of the corresponding turtle host was therefore not used in statistical analyses, and instead only host FP status was considered.

Among 2119 turtle captures within the IRL between January 2006 and December 2018, leech prevalence was significantly higher in green turtles compared to loggerhead turtles ($\chi^2_{1,N=2119}$ = 154.85, $p < 0.00001$). Overall, 38.5% of green turtles and 7.9% of loggerhead turtles were captured with at least 1 larval or adult leech attached. Similarly, 48.0% of green turtles had FP tumors, while only 10.5% of loggerhead turtles had FP tumors at the time of capture, a significant difference ($\chi^2_{1,N=2119}$ = 210.39, $p < 0.00001$). Comparing FP and leech parasitism simultaneously, 43.7% of FP-positive green turtles had leech parasites compared to 33.7% of FP-negative individuals. In loggerhead turtles, 10.4% of FP-positive individuals had leeches compared to 7.5% of FP-negative individuals.

Using GAMs, we found that none of the covariates tested (host FP status, SCL, BCI, and seasonality) were significantly correlated to leech presence in loggerhead turtles. In contrast, FP status and month of capture, but not SCL or BCI, were significant predictors of leech presence in green turtles. Green turtles with FP were more likely to have at least 1 larval or adult leech than FP-negative individuals (Z = 3.436, $p < 0.001$) (Fig. 2). Green turtles were most likely to have at least 1 larval or adult leech when captured in the winter and least likely in the summer ($\chi^2_{7.007,N=1676}$ = 120.6, $p < 0.0001$) (Fig. 3). A similar significant pattern of seasonal prevalence was seen in leech eggs on green turtles ($\chi^2_{7.597,N=1676}$ = 80.94, $p < 0.0001$) (Fig. 3).

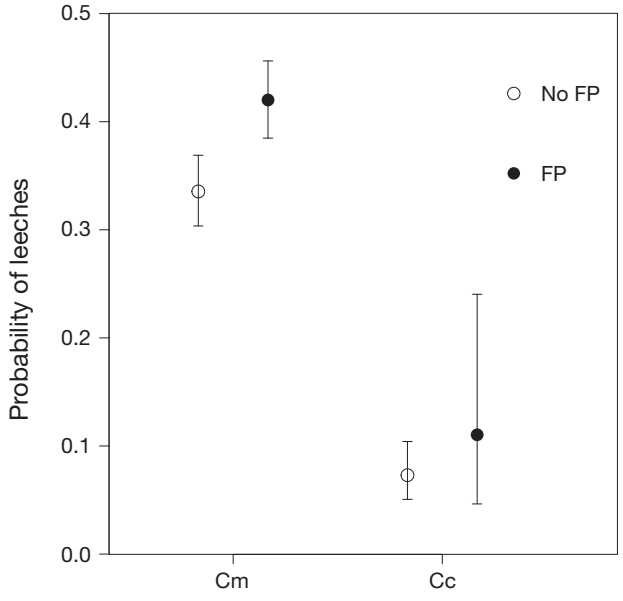

Fig. 2. Generalized additive model with 95% CIs for the probability of a larval or adult leech being found on green turtles *Chelonia mydas* (Cm) and loggerhead turtles *Caretta caretta* (Cc) with and without fibropapillomatosis (FP) between 2006 and 2018 in the Indian River Lagoon, Florida, USA. Green turtles with FP were more likely to have at least 1 leech than those without FP ($Z = 3.436$, $p < 0.001$)

4. DISCUSSION

Many aspects of the epidemiology of sea turtle FP remain uncertain, including the potential presence of a vector species. Two species of the leech genus *Ozobranchus* can harbor significant viral loads of the FP-associated pathogen ChHV5 and have been proposed as mechanical vectors (Greenblatt et al. 2004), a pattern we confirm here with our sampled leeches. However, studies on differences between the 2 leech species in terms of their association with FP are minimal (Bunkley-Williams et al. 2008), as are studies comparing leech parasitism among sea turtle species (Sawyer et al. 1975, Bunkley-Williams et al. 2008, McGowin et al. 2011). Here, we demonstrate that leech parasitism in the IRL is significantly correlated with host FP and capture month in green turtles but not in loggerhead turtles. These results suggest that the roles of *Ozobranchus branchiatus* and *O. margoi* in disease transmission and development may differ or that potential differences in host biology may impact susceptibility to FP and leech parasitism.

Within our study site, all 55 sampled *O. branchiatus* were collected from green turtles and all 22 sampled *O. margoi* were collected from loggerhead turtles, consistent with previous studies that detected strong host specificity (Sawyer et al. 1975, Bunkley-Williams et al. 2008, McGowin et al. 2011). Thus, *Ozobranchus* spp. are unlikely to significantly contribute to ChHV5 transmission between species. In contrast, *Ozobranchus* spp. may play a significant role in the intraspecific transmission of ChHV5. Numerous species of leeches across different orders act as intraspecific vectors of viral pathogens (Shope 1957, Ahne 1985), suggesting parasitic sea turtle leeches may also be capable of vectoring disease.

We found ChHV5 viral loads within *Ozobranchus* spp. up to 17 times the loads found in our positive control tumor sample. However, because we did not quantify ChHV5 viral copies per cell in our leech samples, we could not statistically compare leech viral loads to known FP tumor viral loads (Quackenbush et al. 2001). In green turtles, ChHV5-positive leeches significantly trended towards coming from hosts with FP, though our statistical analyses provided limited insight due to sample size restrictions. More robust analyses with larger sample sizes would prove valuable in future studies investigating relationships between leech ChHV5 viral load and turtle FP. These data continue to be absent in loggerhead turtles. Additionally, it remains uncertain whether associations between ChHV5-positive leeches and FP would indicate viral transmission from a ChHV5-positive leech to an unaffected turtle or transmission from an FP-positive turtle to a previously ChHV5-nega-

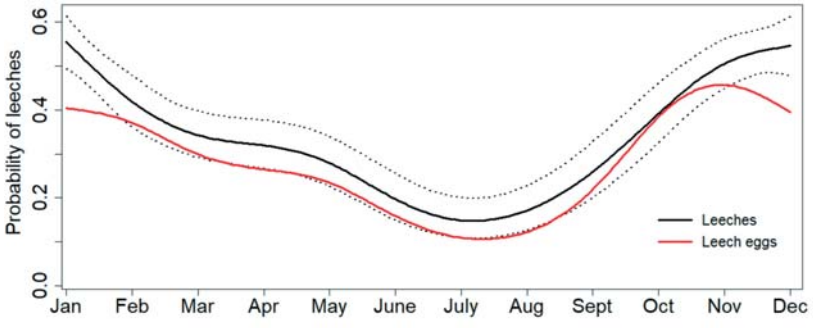

Fig. 3. Generalized additive models for the monthly probabilities of larval or adult leeches (black line) and leech eggs (red line) being found on juvenile green turtles between 2006 and 2018 in the Indian River Lagoon, Florida, USA. Black dotted lines indicate 95% confidence intervals for larval or adult leeches. There was a seasonal trend in leech presence for both life stages, where leeches ($\chi^2_{7.007,N=1676} = 120.6$, $p < 0.0001$) and eggs ($\chi^2_{7.597,N=1676} = 80.94$, $p < 0.0001$) were most likely to be found on green turtles in the winter and least likely in the summer

tive leech. Experimental ChHV5 transmissions using leeches may help provide these answers.

Of note is that none of the host blood samples tested positive for ChHV5 despite 15 leeches from 8 different hosts testing positive. Circumstances related to sampling, qPCR assay performance (Lawrance et al. 2018), or virus–host dynamics may have prevented the detection of ChHV5 in the blood samples and resulted in false negatives. Internal PCR controls targeting turtle housekeeping genes should be utilized to verify that negative ChHV5 qPCR results do not arise from PCR inhibitors. However, because we successfully amplified host genes for a separate study (A. E. Savage unpubl. data) from the same blood DNA extractions used for ChHV5 qPCR in this study, we can conclude that PCR inhibition does not account for our ChHV5-negative results. The virus could exist in host blood either intermittently or at levels below the qPCR detection limit. The high levels of ChHV5 found in some leeches may be due to the accumulation of virus filtered from host blood over time. Alternatively, leeches could potentially obtain and accumulate ChHV5 through ingestion of host epithelium (Greenblatt et al. 2004). Further work is necessary to determine how leeches obtain ChHV5, and researchers should consider using nested PCR of several viral genes to get better estimates of ChHV5 prevalence and its relationship to FP and leech parasitism (Lawrance et al. 2018).

From our dataset of 443 loggerhead turtle capture events, we found no significant relationship between leech parasitism and host FP. Because the large majority of reported leeches on loggerhead turtles are *O. margoi* (reviewed in Bunkley-Williams et al. 2008), and because we found that host FP status was not significantly correlated to leech presence on loggerhead turtles, *O. margoi* is unlikely to play a significant role in FP development. Thus, the role of *O. margoi* as a ChHV5 vector may be less likely than previously thought (Greenblatt et al. 2004). However, ChHV5 can exist in a latent state in hosts without clinical signs of FP (Greenblatt et al. 2004, Page-Karjian et al. 2015); therefore, leeches may be vectoring the virus independent of tumor formation. Because Ozobranchidae leeches are semi-permanent residents of turtles (Sawyer et al. 1975), a leech may leave a host after transmitting ChHV5 but before FP develops. In captivity, *O. margoi* infestations can quickly spread from turtle to turtle, suggesting that *O. margoi* may not stay on 1 host for long under certain circumstances (Schwartz 1974). If *O. margoi* stays on 1 host for an extended period of time and significantly contributes to ChHV5 transmission, then we would predict a significant correlation between loggerhead turtles with FP and leech parasitism. Fundamentally, we need more research on the life history of *O. margoi* and its ability to carry ChHV5 before we can robustly assess the role of these leeches in viral vectoring and other relationships with FP.

Despite the lack of correlation between FP status and leech presence on loggerhead turtles, green turtles with FP were significantly more likely to have at least 1 larval or adult leech than green turtles without FP. To the best of our knowledge, this study is the first to provide robust statistical evidence of a significant association between leech presence and FP in green turtles using a large sample size. Because leech presence was not significantly correlated to BCI, the relationship between FP and leech parasitism is not likely explained by poor overall health of the host resulting in high susceptibility to both FP and ectoparasites. Instead, our results suggest that *O. branchiatus* parasitism may play a role in FP development or that it is significantly attracted to turtles with FP, as suggested by Nigrelli & Smith (1943) and Ehrhart (1991), respectively.

FP tumors may improve the ability of a leech to locate a potential host, or leeches may be less likely to leave a host that is FP positive. Whether *Ozobranchus* spp. can detect FP is unknown, though chemoreception is suspected in the 2 other families in Rhynchobdellida, the paraphyletic order that contains Ozobranchidae (Khan & Emerson 1981, Moser et al. 2009). Tumors associated with FP often have highly vascularized regions (Burkhalter & Norton 2019), which would provide a ready supply of blood to the hematophagous leech and could be an advantage of encountering and remaining on an FP-positive host. If *O. branchiatus* is more likely to encounter and remain on FP-positive turtles than on FP-negative turtles, while *O. margoi* is not, this may explain the difference in association between leeches and FP on green and loggerhead turtles. It is also possible that green and loggerhead turtles differ in how FP manifests, possibly impacting the potential chemoreceptive abilities of *O. margoi* and *O. branchiatus* or the advantages of parasitizing an FP-positive host.

Alternatively, host immune status may explain the significant association between leech presence and FP in green turtles. Ectoparasites often deploy mechanisms to suppress the host's immune response and may preferentially parasitize immunocompromised hosts (Kerlin & East 1992, Roulin et al. 2003). Green turtles with FP often are immunosuppressed and anemic and have lower rates of phagocytosis

(Aguirre et al. 1995, Work et al. 2001, Sposato 2014), possibly allowing leeches to better establish and persist on FP-positive hosts. Data comparing the immune systems of FP-positive and FP-negative loggerhead turtles are lacking. However, healthy captive loggerhead turtles and green turtles differ in their blood composition and cytochemical characteristics (Casal & Orós 2007), which may result in different levels of immunosuppression between FP-positive loggerhead and green turtles. This may explain why leeches were significantly associated with FP in green turtles but not in loggerhead turtles. *O. margoi* and *O. branchiatus* may differ in how they impact the host immune system, or loggerhead and green turtles may vary in their responses to FP and leeches. Additionally, the impact of FP on turtle health may affect the turtle's ability to remove ectoparasites by self-cleaning or posting at stations with cleaner reef fish (Losey et al. 1994, Schofield et al. 2006). However, our study site is not a reef system and is not currently known to have any cleaning stations (Ehrhart et al. 2007). We also failed to recover a relationship between host body condition and leech parasitism, suggesting that an inability to post at cleaner stations due to poor health is not a significant contributor to leech parasitism at our study site.

Host immunity may also play a role in the seasonal variation in leech presence in green turtles. Capture month was a significant predictor of leech presence in green turtles, with the highest probability of leech parasitism in winter months and the lowest probability in summer months. No seasonal pattern was observed in loggerhead turtles. Green turtles from the IRL have higher rates of phagocytosis in the summer than in the winter, though rates are independent of temperature in *in vitro* experiments (Sposato 2014). There is no difference in rates of phagocytosis in loggerhead turtles from the IRL across seasons (Sposato 2014). Decreased host immunity in the winter may lead to increased parasitic load, which may explain why a seasonal trend in leeches was seen in green turtles but not in loggerhead turtles.

Because leech presence could not be predicted by host SCL, variation in leech presence on green turtles throughout the year is likely not tied to seasonal fluctuations in the IRL of average turtle SCL likely caused by the recruitment of juveniles (Ehrhart et al. 2007). Because our models recovered separate effects for FP status and capture month on green turtle leech prevalence, seasonal trends in FP alone cannot explain variations in leech presence. We also recovered similar patterns of seasonal variation between leech eggs and larval and adult leech prevalence. This suggests that green turtle leeches do not exist primarily as eggs or cocoons during the summer and adults during the winter, as seen in other aquatic leech species (Allen & Allen 1981). While all life stages of *Ozobranchus* spp. are hypothesized to occur on turtle hosts (Sawyer et al. 1975), it is possible that a currently unknown aspect of the life history of *O. branchiatus*, such as leaving the turtle host in warmer months, may explain the seasonal variation in presence seen in the IRL. If *O. branchiatus* is less tolerant of warmer temperatures or mainly attaches to hosts in the colder months, as seen in other aquatic leech species (Allen & Allen 1981), observed populations may decrease in the summer.

Seasonal variation in turtle movement may also contribute to observed temporal trends in leech presence. In the Mosquito Lagoon, just north of our study site, green turtle vagility increases during winter months (Mendonça 1983). Similarly, catch per unit effort (CPUE) rates increase for green turtles in the winter at our study site, suggesting increased movement into and within the IRL during the winter (Ehrhart et al. 2007). These findings suggest that higher leech prevalence in the winter is not due to decreased movement of sea turtles. This increased movement may, however, increase turtle contact rates and thus the likelihood of leech transmission events. In contrast, loggerhead turtles displayed no variation in CPUE rates across seasons (Ehrhart et al. 2007), which may explain why leech prevalence also did not vary across sampling months.

5. CONCLUSIONS

Future studies should focus on how FP impacts the immune system of loggerhead turtles, the frequency of leech host switching, and possible experimental ChHV5 transmission in sea turtles using leeches. Investigating *Ozobranchus* spp. as vectors of ChHV5 aids in illuminating the epidemiology of FP, and assessing the threat that leeches pose to overall sea turtle health can help to improve sea turtle monitoring, management, and treatment programs.

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