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RESEARCH ARTICLE

Effects of hybridization on sea turtle fitness

Luciano S. Soares[1,2] · Karen A. Bjorndal[1,2] · Alan B. Bolten[1,2] · Maria A. G. dei Marcovaldi[3] · Pedro B. Luz[4] · Rodrigo Machado[5] · Rachel Lo[2] · Stuart F. McDaniel[2] · Adam C. Payton[2] · Thomas B. Waltzek[6] · Marta L. Wayne[2,7]

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Abstract

Sea turtle hybridization is a common phenomenon in Brazil between loggerheads (*Caretta caretta*) and hawksbills (*Eretmochelys imbricata*) as well as between loggerheads and olive ridleys (*Lepidochelys olivacea*). In a previous study we showed that the reproductive output of loggerhead/hawksbill hybrids is similar to that of parental species, suggesting no negative effect of hybridization at this life stage. In this study, we used pooled amplicon sequencing to assign species identity to dams and their progeny, and to investigate the fitness consequences of hybridization, using hatchling viability as a proxy for fitness. We genotyped 4829 hatchlings from egg clutches laid by 78 loggerheads, 13 hawksbills, seven loggerhead/hawksbill hybrids, and three loggerhead/olive ridley hybrids. The proportion of viable hybrid (heterozygous) hatchlings was similar to that of homozygous hatchlings (based on data at two loci), independent of the dam's genotype. Multiple species paternity was observed in 35.7% of the nests. Both hybrid males and females were fertile and produced viable offspring, and we found no evidence for hybrid breakdown. We suggest a genome-wide study of the hybrids and parental species to better characterize hybrids, as well as studies on additional demographic and ecological parameters to further assess the effects of hybridization and its consequences for sea turtles and their environment.

Keywords Hybridization · Sea turtles · Loggerhead · Hawksbill · Olive ridley · Fitness

Introduction

Hybridization among wild taxa is spread across a vast taxonomic range and is recognized as having conservation importance (Stronen and Paquet 2013). Hybridization is usually viewed negatively because of possible fitness declines, for example if there is hybrid inviability, sterility, or even hybrid breakdown (Arnold and Hodges 1995). In addition, interspecific hybridization can lead to the loss of the rarer species due to swamping (Todesco et al. 2016). However, hybridization can also be beneficial and can even result in genetic rescue (Stronen and Paquet 2013).

Whether or not groups of organisms belong to distinct species, versus representing a single population with phenotypic variants, has particular importance for species of conservation interest given the wording of conservation law, such as the Convention on International Trade in Endangered Species (Article I), the Endangered Species Act (Sect. 3(15)) in the United States of America and the Brazilian Endangered Species List. Conservation policies must be inclusive and encompass wild populations of hybridizing animals in the context of their ecological roles (Stronen and

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✉ Luciano S. Soares
lsoares@ufl.edu

[1] Archie Carr Center for Sea Turtle Research, University of Florida, PO Box 118525, Gainesville, FL 32611, USA

[2] Department of Biology, University of Florida, Gainesville, FL, USA

[3] Projeto TAMAR and Fundação Pró-TAMAR, Salvador, BA, Brazil

[4] Departamento de Ecologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[5] Centro de Ciências do Mar, Universidade do Algarve, Faro, Portugal

[6] Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA

[7] University of Florida Genetics Institute, University of Florida, Gainesville, FL, USA

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Paquet 2013). Developing optimum conservation strategies for hybridizing populations requires understanding of various facets involved in the process such as reproductive output, fitness, survivorship, genetic incompatibilities and ecological roles. With the intent to increase the knowledge on hybrid dynamics, we sampled sea turtles and their hybrids from the same population studied by Soares et al. (2017), to associate complementary data on hatchling viability with the data previously reported on hybrid reproductive output.

Sea turtles are iconic species for marine conservation and moreover maintain habitats important for other species, as they perform their roles as ecosystem engineers (Bjorndal and Jackson 2003). The seven species of sea turtles now identified are well-recognized species (based on morphology and molecular markers) that occasionally hybridize. Among the Chelonidae, hybridization was reported for the first time in 1888 (Garman 1888) based on the morphology of a probable cross between a loggerhead (*Caretta caretta*) and a hawksbill (*Eretmochelys imbricata*). Since then, there have been multiple observations of hybridization for almost all species within the Cheloniidae family (Bowen and Karl 2007; Reis et al. 2010; Vilaça et al. 2012; Kelez et al. 2016; Soares et al. 2017). The incidence of hybridization is not known for most populations, but given the relatively low number of reports of hybrids from most areas, it is thought to be very low with the exception of populations nesting in Brazil.

In Brazil, a hybrid loggerhead/hawksbill was first reported by Conceicao et al. (1990). Subsequently, hybrids between all combinations of loggerheads, hawksbills and olive ridleys (*Lepidochelys olivacea*) have been reported on Brazilian nesting grounds in the states of Bahia and Sergipe (Bass et al. 1996; Lara-Ruiz et al. 2006; Reis et al. 2010; Vilaça et al. 2012; Soares et al. 2017). Lara-Ruiz et al. (2006) reported an incidence of 42% of hybridization between loggerheads and hawksbills in the 119 females morphologically assigned as hawksbills in Bahia, the main nesting site for this species in Brazil. Reis et al. (2010) observed 27% of loggerhead/olive ridley hybrids in the morphologically assigned loggerheads ($N = 51$) in the neighboring state of Sergipe, an important loggerhead nesting area, and the major olive ridley rookery in Brazil. These studies were based on adult nesting females, that are expected to be at least 30 years old, and thus reflect hybridization events that occurred long ago. In the 1980s, approximately 30 years before the study of Reis et al. (2010), Projeto TAMAR-ICMBio (the Brazilian Sea Turtle Conservation and Research Program) began its conservation efforts in response to an ongoing decline among Brazilian populations of loggerheads, hawksbills and olive ridleys. Thus, one possible explanation for the high hybridization incidence is the reduced numbers of potential conspecific mates, as well as the spatial and temporal overlap of the nesting seasons (Vilaça et al. 2012). Hybridization among sea turtles, as presented above, has been reported for more than 100 years, but the dynamics are poorly understood. Given their conservation interest, it is important to understand the consequences of hybridization such as fitness costs, loss of rare species due to swamping and changes in mating systems. Continued long term studies should be planned to document the dynamics of this phenomenon through time.

In this paper, we explore whether interspecific hybridization has fitness consequences for these three species of sea turtles. We genotyped 101 nesting females and a sample of their offspring (N = 4829) to determine hybrid status, and quantified the viability of hybrid and non-hybrid hatchlings as a proxy for fitness. This proxy does not take into account the many other potential consequences of hybridization, such as survivorship in other life stages, growth rates, mating success or breeding phenology. Observed fitness differences would be evidence of reproductive isolation and could also act as a selective force promoting conspecific recognition (i.e., reinforcement [Butlin 1987]). We also investigated the direction of crosses, whether individual nests were sired by multiple species (multiple paternity within species is common in sea turtle clutches), and if hybrid males were fertile.

Methods

Data collection and sampling

We collected a total 4930 tissue samples from 101 nesting females and their offspring. These were collected in the two main nesting rookeries for loggerheads and hawksbills in Brazil, Praia do Forte and Arembepe (Fig. 1). Between October 1st 2012 and April 15th 2013, we patrolled the nesting areas from 20:00 to 05:00 to encounter nesting females and emerging hatchlings and to excavate hatched nests to collect samples from unhatched eggs and dead hatchlings.

We morphologically identified sampled females as 82 loggerheads and 19 hawksbills. We collected tissue samples from nesting females between the first and second scales on the front flippers or from the neck region with a 6-mm biopsy punch and stored the tissue in 70% alcohol. All females were double tagged (National Band and Tag Co., style 681) (Marcovaldi and Marcovaldi 1999) to avoid re-sampling. We monitored each nest until hatchling emergence after approximately 50 days. We placed a circular fence on top of the nest approximately 5 days before emergence, after which the nests were checked every half hour from (17:30 to 07:00) until the hatchlings surfaced. Tissue from live hatchlings (N = 2424), from now on referred to as "viables", was collected from the tip of the last marginal scute in the carapace using a sterile disposable blade, removing a 2-mm tissue fragment.

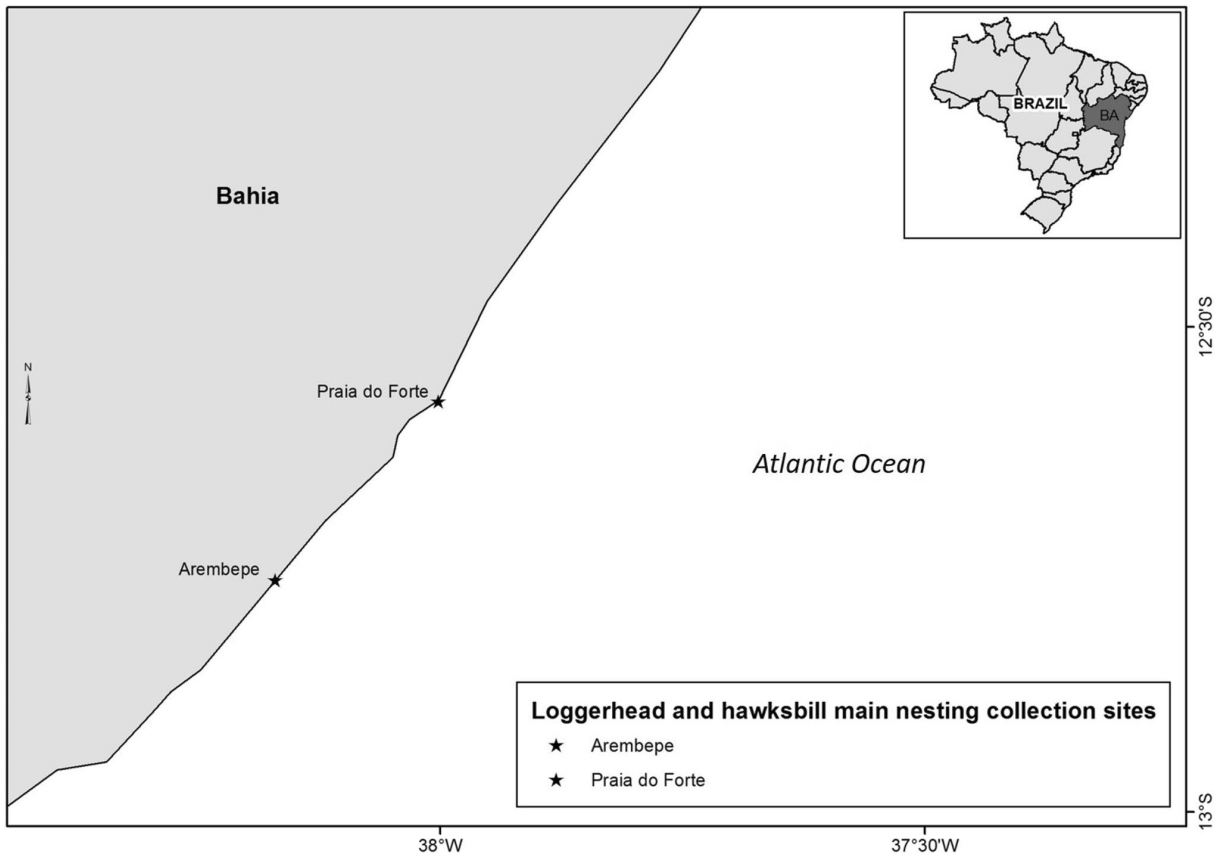

Fig. 1 Map indicating sample collection sites on main loggerhead and hawksbill nesting sites in Brazil

Hatchlings were released to the nesting beach within at most 30 min after sampling. We also sampled (N = 2405) dead hatchlings and embryos, from now on "non viables", the next morning following nest excavation. Samples were collected with a sterile disposable blade, and size varied with the available size of the embryo. Samples varied from a blood drop to a whole flipper. All samples were stored in 10 ml 95% ethanol in 20 ml disposable scintillation vials.

Genetic analyses and species assignment

All adult females and hatchlings were assigned as either loggerheads, hawksbills or hybrids based on the combined use of mtDNA and a nuclear marker (RAG2, as described in Vilaça et al. 2012). We first extracted genomic DNA using a DNeasy Blood and Tissue Kit (QIAGEN Inc.) following the manufacturer's protocol. Genetic data were collected by both Sanger and NGS (Next Generation Sequencing) techniques, as described in more detail below.

Sanger sequencing and assignment

We amplified a ~ 830 bp fragment of the mtDNA encompassing the D-loop of the control region and the adjacent $tRNA^{Thr}$ and $tRNA^{Pro}$ with primers LCM15382 and H950 developed by Abreu-Grobois and colleagues, as cited in Proietti et al. (2014). We conducted 25 µl PCR reactions which included 50 ng of genomic DNA, 12.5 µl of NEB One Taq Hot Start Master Mix (M0488L, New England Biolabs, Inc.), 9.5 µl of sdH_20 and 1 µl of each primer, with the following PCR conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 50.5 °C for 60 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. After amplification, the PCR products were confirmed by running 1.5% agarose gels stained with GelRed. The PCR products were purified using ExoSAP-IT (USB Corporation) according to the manufacturer's instructions. Samples were Sanger sequenced for both strands using both amplification primers at the DNA Analysis Facility at Yale University run in the Thermo Fisher Scientific 96-capillary 3730xl DNA Analyzer.

We amplified and Sanger sequenced four species-specific autosomal markers previously used by Vilaça et al. (2012)

(RAG1, RAG2, R35 and CMOS) for 25 nesting females morphologically assigned as 15 loggerheads and 10 hawksbills. We conducted PCR reactions as described above with the following PCR cycling parameters for RAG1 and R35: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 61.7 °C for 60 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. The PCR cycles for RAG2 were: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 64.4 °C for 60 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. Finally, for CMOS the parameters were: 95 °C for 15 min, 35 cycles of 95 °C for 30 s, 66 °C for 60 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. After amplification, PCR products were confirmed by running 1.5% agarose gels stained with GelRed. PCR products were then purified using ExoSAP-IT (USB Corporation) according to the manufacturer's instructions. Samples were Sanger sequenced for both strands using either of the amplification primers at the DNA Analysis Facility at Yale University.

We established that there were no discordances among the resulting assignments for the four nuclear markers and the mitochondrial marker for 25 females. We then selected a single nuclear marker for future species assignments. Given the assignment accuracy of 99.9% for 327 turtles based on RAG2 in the paper by Vilaça et al. (2012), the remaining 76 samples were genotyped only for RAG2 (following the same methodology described above). RAG2 contains three SNPs which are combined to create species-specific haplotypes.

NGS sequencing

We used a Next Generation targeted amplicon sequencing approach to individually amplify and barcode the RAG2 PCR product for all 101 females and their 4829 progeny. We used a modification of the Illumina 16S protocol in which the locus of interest is amplified using gene specific primers containing unique nucleotide tails on their 5′ end. After the first PCR reaction, a second PCR is performed using a second set of primers which are complementary to the tails. These primers contain the Illumina flow cell binding sequence, a unique seven base pair index (Mir et al. 2013) that serves as a barcode, and the binding sequence for the Illumina sequencing primer. By using this two-step PCR approach it allows for the single purchase of the long indexing primers, used for the second PCR, which are paired with loci specific primers which can be inexpensively purchased for a given project. To obtain enough unique indices for the 4948 individuals, we used a combinatorial approach where a set of 72 index on the 5′ end of the amplicon were paired with 70 3′ indices to provide up to 5040 unique combinations of the two index sets. Gene specific primers and their associated tails were designed following the Illumina 16S protocol (15044223 Revision B), and indexing primers were modeled after Illumina Nextera sequence adaptors (1000000002694 v00 Oligonucleotide Sequences © 2015 Illumina, Inc). Indices were seven base pairs long with no homopolymer runs and were 3 bp different from any other index (Mir et al. 2013).

PCR1 was performed using NEB TAQ (M0488l, New England Biolabs, Inc.) in 25 µl reactions under the following thermocycler (Master Cycler Pro S, Eppendorf Inc.) conditions (94 °C for 30 s, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 60 s and a final extension of 68 °C for 5 min). PCR 2 was performed using NEB TAQ (M0488l, New England Biolabs, Inc.) in 12 µl reaction volumes under the following thermocycler conditions (94 °C for 30 s, 12 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 630 s and a final extension of 68 °C for 5 min). Following PCR2, 5 µl of PCR product was visualized using agarose electrophoresis to confirm successful amplification and incorporation of both indexing adaptors. 5 µl of PCR product was combined for all samples and run through Qiagen PCR clean up Columns (QIAGEN PCR Purification Kit, Inc.) following manufacturers' protocol to remove enzymes and unincorporated nucleotides, and to concentrate the final samples. DNA concentrations were calculated prior to column clean up to ensure each column's maximum DNA binding capacity was not exceeded. Sufficient columns were used to ensure the cleanup of the pooled PCR products for all individuals. Prior to sequencing, the pooled sample was analyzed for the presence of unincorporated adaptors and primers using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and sequenced on an Illumina MiSeq 2×300 v3 chemistry at the Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida. Sequence reads were demultiplexed, allowing 1 bp of mismatch at the index, on the MiSeq instrument as part of the Illumina post sequencing data processing. All reads were trimmed for quality using NGSUtils (Breese and Liu 2013).

Species assignment

For mtDNA assignments, we aligned haplotypes for each marker against known loggerhead and hawksbill haplotypes as previously determined by the Archie Carr Center for Sea Turtle Research (http://accstr.ufl.edu/files/accstr-resources/cclongmtdna.pdf) and Vilaça et al. (2012). We used the software Geneious R8 (Kearse et al. 2012) with default Geneious Alignment algorithm parameters. Heterozygotes were assigned when the chromatograms for the nuclear markers clearly showed the presence of both species' alleles at all diagnostic polymorphic nucleotides within the locus for each gene.

To process the NGS reads and call SNPs, custom Perl scripts were used to search each individual's processed Illumina reads, identify SNPs present for species determination, and make homozygosity or heterozygosity/hybridization

calls. To determine a false positive detection rate, individuals of known species determination (i.e., pure loggerhead or hawksbill) were identified based on the cumulative evidence of morphology, additional nuclear markers, and mitochondrial markers. The quantity of reads found in each of these individual's NGS data that did not support the species designations was determined. Based on these data we determined 17 reads was the minimum number of reads necessary for a given SNP to allow 95% confidence in the species assignment of that individual. The criteria for the Perl script based homozygosity and heterozygosity calls is as follows: Based on our false positive threshold, all individuals which had fewer than 17 reads supporting an allele call were discarded. An individual was considered homozygous when 90% or more of the sequence reads were homozygous for a given species' diagnostic SNPs. Heterozygotes, representing hybrids or potentially backcrosses, were identified when the diagnostic alleles for both species were each present in a minimum of 40% of the total reads. This threshold was used to identify the individuals in which each allele closely matched the 50:50 theoretical allelic ratio for a F1 hybrid. This threshold was then lowered to allow for the detection of alleles that were present in 25–39% of the total alleles, allowing for the identification of heterozygous individuals that have good support based on sequence coverage but are farther from the theoretical 50:50 allelic ratio. If individuals showed support for heterozygosity but the minor allele was less than 25% but greater than 10% of the total reads, those individuals were flagged and examined more carefully. Heterozygosity for these individuals was assigned only if additional progeny from the same nest also were heterozygote for the same SNPs but were called with greater support (e.g., the minor allele was present in > 25% of the reads). If no additional progeny showed evidence for heterozygosity, the individual was discarded. We consider this a conservative approach that ensures a nest is not considered of hybrid origin unless there are multiple observations each with high support.

Results

We conducted genetic analyses of 101 dams and 4829 of their progeny collected in Bahia, Brazil, in the 2012/2013 nesting season to assess incidence and consequences of interspecific hybridization in hawksbill and loggerhead sea turtles. We used one mtDNA marker and one nuclear locus (RAG2) on the dams and hatchlings. Thus, we were able to assign species identity as well as maternity (i.e., the direction of any interspecific crosses) for all individuals.

Because we used only a single locus with three SNPs, in general we do not have the ability to detect certain recombinants or complex backcross progeny. Thus, we refer to individuals as either homozygotes (alleles of only one species present) or heterozygotes (alleles of more than one species present). To describe a mating, we use the terms "conspecific" for cases where only alleles from a single species were detected in hatchlings from the nest, and "heterospecific" for the alternative (i.e., alleles from more than one species were detected in the nest). We also use the term "hybrid" to denote progeny that are either themselves heterozygous, or are the progeny of a heterozygous dam. Depending on the sire, the progeny of heterozygous dams may be heterozygous, but may also be homozygous backcross progeny.

Hybridization incidence, mate choice and seasonality

To assess historical incidences of hybridization (i.e. inferred from nesting females; Table 1), we assessed the data available from genetic assigned loggerheads, hawksbills and

Table 1 Incidence of hybridization in nesting females

Paper	Morphological species	Genotype	Hybridization incidence (%)
Reis et al. (2010) (SE)	51 (loggerheads)	14 (OL[a])	27.45
		37 (LL)	
Lara-Ruiz et al. (2006) (BA)	119 (hawksbills)	50 (LH[b])	42.02
		69 (HH)	
This study (BA)	82 (loggerheads)	01 (HL[c])	1.22
		03 (OL[a])	3.66
		78 (LL)	
	19 (hawksbills)	06 (LH[b])	31.58
		13 (HH)	

HH hawksbill, *LL* loggerheads, *LH* loggerhead/hawksbill hybrid with loggerhead mtDNA (loggerhead dams[b]), *HL* hawksbill/loggerhead hybrid with hawksbill mtDNA (hawksbill dam[c]), *OL* olive ridley/loggerhead hybrid with olive ridley mtDNA (olive ridley dams[a]), *BA* samples collected in Bahia state, *SE* samples collected in Sergipe state

hybrid females nesting in Bahia. We found that the hybridization incidence of loggerhead dams from this study is 4.88%, N = 82 (Table 1). Three out of the four hybrid females assigned as morphological loggerheads in our study were loggerhead/olive ridley hybrids (Table 1). Only one morphologically identified loggerhead was a loggerhead/hawksbill hybrid. In contrast, six out of 19 morphologically identified hawksbills were loggerhead/hawksbill hybrids (Table 1).

To assess the percentage of hybrid hatchlings that have a chance of recruiting to the population, we used only the data from viable hatchling genotypes (live hatchlings). The percentage of hybridization inferred from viable hatchlings from both homozygous and heterozygous dams is 21.62% (N = 2105, Table 2). For nests of loggerhead dams, this incidence was 16.66% (N = 1777, Table 2), while for hawksbill dams it was 8.15% (N = 184, Table 2).

To assess if a nest was sired by males of more than one species (multiple species paternity) we used direct inference based on dam and hatchling genotypes (see Supplementary S1—Table 1). Multiple species paternity was observed in 35.7% of all heterospecific nests (10 of 28, Table 3). For loggerhead heterospecific nests (i.e., nests of loggerheads that had heterozygous offspring), multiple species paternity was observed in 47% (7 of 15; Table 3). Because we have only one nuclear marker, we cannot always perfectly infer the sire's genotype from those of the dam and the progeny. However, the data suggest that some of the loggerhead females were likely mating with hawksbill, olive ridley, loggerhead/hawksbill, loggerhead/olive ridley and/or hawksbill/olive ridley males as well as with loggerhead males (47%; Table 3). Single species paternity was observed in the remaining 53% of heterospecific loggerhead nests. In these eight nests, the total number of offspring was 374, with a median percentage of hybrid offspring of 56.7. This latter group of loggerhead females had mated either with loggerhead/hawksbill males (37.5%), loggerhead/olive ridley males (37.5%) or with hawksbill males (25%).

Although we only observed three heterospecific hawksbill nests, these appeared to have different patterns of hybridization from the loggerhead nests. For example, no instances of multiple species paternity were observed. Also, for two of the nests, only a single hybrid offspring was detected in each. In one case (total offspring = 29), the hybrid was sired by a loggerhead; in the other (total offspring = 71), the hybrid was sired by an olive ridley. In the third nest, only hybrid offspring were detected (N = 15, all hawksbill/loggerhead),

Table 2 Relationship between hatchling genotypes and viability

Hatchling genotype	Dam genotype	V	NV	Incidence of viable hatchling genotype (%)	Incidence of combined (V and NV) hatchling genotype (%)
HH	HH	169	344	8.03	12.90
	LH	13[a]	68	0.62	2.04
HO	HH	0[a]	1	0.00	0.03
	LH	4[a]	5	0.19	0.23
	LO	13[a]	7	0.62	0.50
LL	LL	1481	1091	70.36	64.69
	LH	15[a]	21	0.71	0.91
	LO	27[a]	18	1.28	1.13
LH	HH	15[a]	2	0.71	0.43
	LL	209[a]	148	9.93	8.98
	LH	18[a]	76	0.86	2.36
	LO	15[a]	8	0.71	0.58
LO	LL	87[a]	54	4.13	3.55
	LH	7[a]	9	0.33	0.40
	LO	32[a]	18	1.52	1.26
LHO	HH	0[a]	0	0.00	0.00
	LL	0[a]	0	0.00	0.00
	LH	0[a]	1	0.00	0.03
	LO	0[a]	0	0.00	0.00

V viable hatchlings, *NV* non-viable hatchlings, *Combined V and NV* sum of V and NV, *LL* homozygous loggerhead, *LH* loggerhead/hawksbill heterozygote, *HH* homozygous hawksbill, *HO* hawksbill/olive ridley heterozygote, *LO* loggerhead/olive ridley heterozygote, *LHO* loggerhead/hawksbill/olive ridley (because the RAG2 locus was ~300 bases long and contained three informative SNPs, in some rare cases we were able to detect recombinants between SNPs). The parent of origin is not specified by the order of the letters

[a]Viable hybrid hatchlings (heterozygotes and homozygotes from heterozygote dams) = 21.62%

Table 3 Multiple species paternity for heterospecific nests

Dam genotype	# Hatchlings of each genotype/nest							Multiple species paternity	Possible sire genotypes
	LL	LH	HH	HO	LHO	LO	Total		
LL	3	53	0	0	0	0	56	Yes	HH, LH, LL
	64	2	0	0	0	0	66	Yes	HH, LH, LL
	70	1	0	0	0	0	71	Yes	HH, LH, LL
	1	41	0	0	0	0	42	Yes	HH, LH, LL
	87	0	0	0	0	1	88	Yes	LL, LO, OO
	36	0	0	0	0	6	42	Yes	LL, LO, OO
	20	18	0	0	0	2	40	Yes	LL, HO, HH, OO
	5	20	0	0	0	0	25	No	LH
	31	22	0	0	0	0	53	No	LH
	22	12	0	0	0	0	34	No	LH
	0	67	0	0	0	0	67	No	HH
	0	41	0	0	0	0	41	No	HH
	29	0	0	0	0	33	62	No	LO
	23	0	0	0	0	28	51	No	LO
	17	0	0	0	0	24	41	No	LO
HH	0	1	28	0	0	0	29	Yes	HH, LH, LL
	0	0	70	1	0	0	71	Yes	HH, HO, OO
	0	15	0	0	0	0	15	No	LL
LH	21	1	32	0	0	0	54	Yes	HH, LH, LL
	10	0	0	0	0	0	10	No	LL
	18	0	15	0	0	0	33	No	HH
	9	0	16	0	0	0	25	No	HH
	14	0	14	0	0	0	28	No	HH
	15	22	0	9	1	16	63	No	LO
	7	13	4	0	0	0	24	No	LH
LO	0	23	0	20	0	0	43	No	HH
	18	0	0	0	0	21	39	No	LL
	27	0	0	0	0	29	56	No	LL

LL loggerhead, *LH* loggerhead/hawksbill, *HH* hawksbill, *HO* hawksbill/olive ridley, *LHO* loggerhead/hawksbill/olive ridley, *LO* loggerhead/olive ridley. The parent of origin is not specified by the order of the letters

which is consistent with this nest being single paternity and the result of a hybrid mating in this generation (Table 3).

We were also able to infer paternity for the progeny of hybrid females (Table 3). For loggerhead/hawksbill dams (N = 7), five nests were most likely sired by a single species. Among these five nests, three were likely sired by hawksbills, the fourth by a loggerhead/hawksbill, and the fifth nest by a loggerhead/olive ridley male given that we saw evidence of three alleles present (loggerhead, hawksbill and olive ridley). In the sixth nest, we saw loggerhead, loggerhead/hawksbill, and hawksbill progeny, a pattern qualitatively similar to an F_2 cross (i.e., a combination of offspring genotypes best explained by two hybrids mating together). Another explanation for this event could be mating with multiple males, one from each of the two parental species. In the seventh nest, we could not determine paternity status due to the small sample size.

For loggerhead/olive ridley females, two out of three nests show no evidence of multiple species paternity and are consistent with a loggerhead sire. The third nest contains both hawksbill/olive ridley and loggerhead/hawksbill offspring, and thus was most likely sired by a hawksbill.

To assess if there was seasonal patterning in heterospecific mating, and in particular to assess whether or not there was any evidence that heterospecific mating might be associated with a paucity of conspecific mates, we plotted nest distribution and type (i.e., conspecific versus heterospecific) for each female genotype (Supplementary Figure 1). Loggerhead conspecific nests are distributed from October through February with peaks in January. Loggerhead heterospecific nests containing either hawksbill or olive ridley alleles also occurred from October to February. Thus, there were no obvious differences in timing of nesting for loggerhead females in conspecific or heterospecific mating. Hawksbill

conspecific nests were only seen in January and February, while heterospecific nests were observed in December and January. Loggerhead/hawksbill females' nests were observed from December through February and loggerhead/olive ridley females' nests were seen only in January and February.

Fitness consequences of hybridization

To compare the effect of hybridization on hatchling viability, we used a X^2 test to assess if there were differences in the number of live pure hatchling and live hybrid hatchlings among all dam types. We also ran the same analyses for each dam type to look at how hybridization affects viability according to the dams' genotype (Table 2). There are 1650 viable homozygote and 1435 non-viable homozygote hatchlings from homozygous dams (loggerheads = 77, hawksbills = 10), and 455 viable hybrid (homozygotes and heterozygotes) and 436 non-viable hybrid hatchlings (homozygotes and heterozygotes; Table 2). Hybridization had no effect on the viability of offspring (N = 3976, $X^2 = 1.5273$, $P = 0.2165$, DF = 1). Considering each species separately, for loggerhead dams, hybridization again had no effect on the number of viable versus non-viable hatchlings (N = 3070, $X^2 = 0.51592$, $P = 0.4726$, DF = 1, Table 2). In contrast, for hawksbill dams, homozygous hatchlings had lower viability than heterozygous hatchlings (N = 531, $X^2 = 17.338$, $P = 3.129^{e-05}$, DF = 1, Table 2). However, we caution that this result may be driven by a small sample size (three nests), which moreover includes one nest in which all genotyped offspring were viable hybrids.

Similar to the loggerhead dams, neither loggerhead/hawksbill (N = 237, $X^2 = 2.8053^{e-31}$, $P = 1$, DF = 1, Table 2) nor loggerhead/olive ridley (N = 138, $X^2 = 0.10702$, $P = 0.7436$, DF = 1, Table 2) hybrid dams revealed any difference in viability between their hybrid homozygous and heterozygous hatchlings.

To assess if hybrid offspring of pure species dams were more viable than hybrid offspring of hybrid females, we compared the viability of loggerhead females' hybrid progeny against the viability of the heterozygote progeny of hybrid loggerhead/hawksbill and loggerhead/olive ridley dams using X^2 tests (Table 4; Fig. 2). We only evaluated loggerheads due to the small sample size (N = 1) for hawksbill dams. The results again suggest no differences (N = 566, $X^2 = 0.1476$, $P = 0.7008$, DF = 1, Table 4; Fig. 2) on the viability of hybrid progeny.

Finally, we were interested in any possible differences in the viability of progeny from dams who appeared to be more selective (only mated with conspecific) relative to dams who were involved in heterospecific matings. We ran a one-way ANOVA on hatchling production (number of live hatchlings that emerge from the nest) comparing loggerhead dams with only conspecific nests (N = 26, mean HP = 71.9) to loggerhead dams with heterospecific nests (N = 15, mean HP = 73.4); there is no significant difference between these two groups (ANOVA, F = 1.007, $P = 0.318$, DF = 1).

Table 4 Comparison of hybrid hatchling viability from homozygous and heterozygous (i.e., hybrid) dams

Dam genotype	Hybrid offspring			
	HomV	HomNV	HetV	HetNV
LL (N = 15)	–	–	211 (56.9%)	160 (43.1%)
HH (N = 1)	–	–	0 (0%)	1 (100%)
LH (N = 3)	14 (12%)	22 (19%)	34 (30%)	45 (39%)
LO (N = 3)	15 (11%)	8 (6%)	72 (52%)	43 (31%)

HomV hybrid viable hatchlings with a homozygote genotype, *HomNV* hybrid non-viable hatchling with a homozygote genotype, *HetV* hybrid viable hatchlings with a heterozygote genotype, *HetNV* hybrid non-viable hatchlings with a heterozygote genotype, *LL* loggerhead, *HH* hawksbill, *LH* loggerhead/hawksbill, *LO* loggerhead/olive ridley. The parent of origin is not specified by the order of the letters

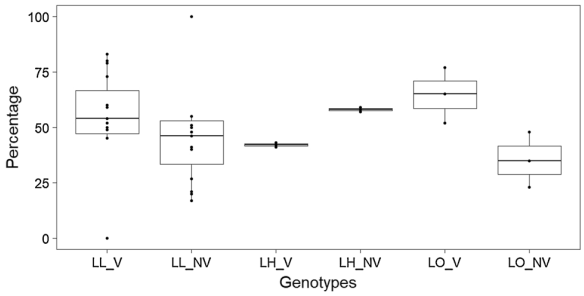

Fig. 2 Box plots comparing viability of hybrid progeny of homozygous dams with two different heterozygous dams. Dam genotypes: *LL* loggerhead homozygotes, *LH* loggerhead/hawksbill hybrids, and *LO* loggerhead/olive ridley hybrids. *V* viables, *NV* non viables. Center lines represent the median, lower and upper lines represent the first and third quartiles (25th and 75th percentiles) respectively

Discussion

Knowledge of sea turtle hybridization is still quite limited. Hybridization has been reported sporadically among juveniles and adult turtles. Very few confirmed hybrid hatchlings have been reported, and no population has been assayed thoroughly for hybridization at the hatchling life stage. In this study, we found that hybrids were common among viable hatchlings (21.62%), and that hatchling viability was indistinguishable between hybrid and non-hybrid offspring. We also detected backcross individuals, which is inconsistent with complete hybrid breakdown. Finally, we detected multiple species paternity, and confirmed that hybrids of both sexes were fertile.

Incidence of hybridization

Lara-Ruiz et al. (2006) observed 42% of adult nesting hybrids among morphologically identified hawksbills in Bahia beaches (N = 119, Table 1). The incidence of hybridization is very similar ($X^2 = 0.74$, P = 0.39, DF = 1) between this study (31.58%, N = 19, Table 1) and Lara-Ruiz et al. (2006) (42.02%, N = 119, Table 1) when considering morphologically identified hawksbills only.

In the neighboring northern state of Sergipe, Reis et al. (2010) identified 27.45% (N = 51, Table 1) hybrid olive ridley/loggerheads among the turtles identified morphologically as loggerheads. In contrast, only 4.88% of the 82 nesting turtles identified morphologically as loggerheads in our study were olive ridley/loggerhead hybrids. Comparing these results to the 27.45% found by Reis et al. (2010) might suggest that hybridization between loggerheads and olive ridleys is declining. However, the difference in this hybridization incidence is likely due to the much greater numbers of olive ridleys nesting in Sergipe, where Reis et al. (2010) did their survey, than our study site in Bahia (Castilhos et al. 2011).

When assessing the incidence of hybridization among hatchlings, we focused on viable hatchlings, as these animals would have a chance of recruiting to the population. Among all viable hatchlings, 21.62% were hybrids (N = 2105). The incidence of all types of hybrid hatchlings from loggerhead dams was 16.66% (N = 1777) and 8.15% (N = 184) from hawksbill dams. The incidence of loggerhead/hawksbill hybrids from loggerhead dams was 9.93% and 0.71% from hawksbill dams. Finally, loggerhead/olive ridley hybrids represented 4.13% of the hatchlings in loggerhead nests.

With these data, we have established a baseline to compare hybridization incidences in this population. Since TAMAR implemented its conservation activities in the 1980s, the loggerhead, hawksbill and olive ridley populations have shown increasing numbers of nests per nesting season. A significant increase in the number of loggerhead nests has been reported by Marcovaldi and Chaloupka (2007), a sevenfold increase in the number of hawksbill nests was reported by Marcovaldi et al. (2007), and, for olive ridleys, nest abundance has increased over ten times (Silva et al. 2007). With greater numbers of individuals in the population, pressure of low conspecific mate availability should be ameliorated. It will be important to revisit hatchling hybridization incidence in the decades to come to establish whether or not hybridization decreases.

Direction of crosses

The incidence of hybridization observed between loggerheads and hawksbills suggests that hybridization is more frequent between loggerhead females and hawksbill males (N = 6) than vice versa (N = 1), and also indicates that male loggerheads can produce viable hatchlings with hawksbill dams. Similarly, the discovery of hatchling loggerhead/olive ridley hybrids from loggerhead dams was very interesting, given that all adult hybrid loggerhead/olive ridleys observed previously were from crosses of a female olive ridley with a male loggerhead (Reis et al. 2010). Our data show both that the reciprocal mating (female loggerheads with male olive ridleys) can occur and that such matings can produce viable progeny.

Given the size differences between loggerheads (male mean curved carapace length = 96.48 cm, N = 64, TAMAR data base; female mean curved carapace length = 99.8 cm, Soares et al. 2017) and olive ridleys (male mean curved carapace length = 67.74 cm, N = 875; female mean curved carapace length = 71.84 cm, N = 3729, TAMAR database), successful reciprocal crosses were unexpected as sea turtle mating behavior involves aggressive male–male competition. One possible explanation could be a lack of loggerhead males when the female loggerheads are mating with olive ridley males, but our seasonality data (Supplementary Figure 1) do not support this hypothesis. Another possible explanation is that there are previously unobserved alternative mating strategies in these species. However, the most likely explanation is that the matings occurred as loggerhead females migrated from the northern foraging areas (Marcovaldi et al. 2007) to the beaches in Bahia, passing through the nesting areas of Sergipe. There is a large olive ridley nesting population in Sergipe, and likely less competition from loggerhead males. In the future, we intend to assess nests of olive ridley dams to analyze what proportion of these would have hybrid individuals.

Hybrids not obviously disadvantaged

Our data show no negative effect of hybridization on hatchling viability, independent of dam or hatchling genotype. Moreover, there is no evidence of hybrid breakdown; viability of the heterozygous progeny of homozygous dams (F1) and heterozygous progeny of heterozygous dams (F2 and/or backcrosses) were statistically indistinguishable (Table 4; Fig. 3).

Both female and male hybrids are fertile and can produce viable progeny (observed by comparing genotypes of viable hatchlings against their dams and possible sires). In addition, we inferred that both loggerhead/hawksbill and loggerhead/olive ridley hybrid males are fertile by use of mitochondrial and nuclear DNA. We observed viable animals from five of the six possible pairwise combinations of the three species, the exception being olive females and hawksbill males. Moreover, we observed two recombinant hatchlings from loggerhead/hawksbill dams, with hawksbill, loggerhead and olive ridley genetic markers. Given that the species-specific

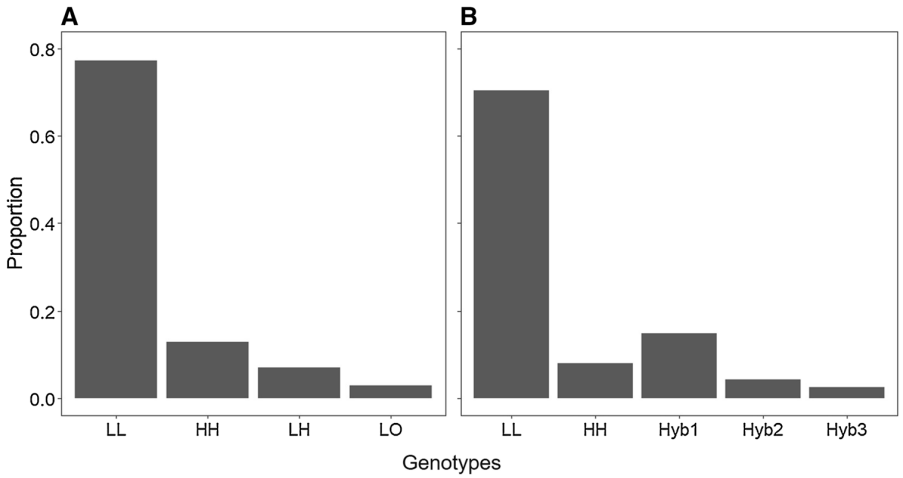

Fig. 3 Comparison of hybridization incidence between dams (**a**) and progeny (**b**). *LL* loggerheads, *HH* hawksbills, *LH* loggerhead/hawksbill hybrids, *LO* loggerhead/olive ridley hybrids, *Hyb1* hybrid progeny (heterozygotes) from a homozygous dam, *Hyb2* hybrid progeny (heterozygotes) from hybrid (heterozygote) dam, *Hyb3* hybrid progeny (homozygotes) from hybrid (heterozygote) dam. Genotypes of hybrid hatchlings are combined in the three hybrid categories presented

SNPs used were tightly linked, this is likely an underestimate of the true multi-species progeny incidence.

Conservation implications

Loggerhead and hawksbill foraging habitats are very distinct (Musick and Limpus 1997; Plotkin 2003). Their diets are also quite different. Loggerheads predominately feed on a variety of benthic invertebrates (including mollusks, crustaceans and sponges), and hawksbills rely primarily on encrusting organisms such as sponges, tunicates, bryozoans (Mortimer 1982; Bjorndal 1997). The different prey species are associated with important morphological differences between the species (i.e., head size, beak morphology). Hybrid loggerhead/hawksbill females (N = 5) satellite tracked by Marcovaldi et al. (2012) shared foraging areas with loggerhead turtles along the northeastern coast of Brazil, but not with adult hawksbills. Juvenile loggerhead/hawksbill hybrids have been observed on loggerhead foraging areas along the south coast of Brazil (N = 3) and in Argentina (N = 2), but none were found on juvenile hawksbill foraging grounds (Vilaça et al. 2013; Proietti et al. 2014; Prosdocimi et al. 2014). These findings show that hybrids may occupy niches similar to those of one but not both of their parental species, experience different selective pressures at different life stages, and respond to selective pressures in distinct ways. To further evaluate whether there are any negative effects of hybridization on these sea turtles, we must understand fitness costs for hybrids at different life stages other than those measured here, such as survival probabilities, growth rates, mating success, and breeding phenology.

The conservation implications of the ubiquity of hybrids in these populations need to be acknowledged, and efforts made to ensure that protection is extended to hybrids as well. Despite mixed morphological traits from both the maternal and paternal species observed in some hybrids (e.g., head size, CCL, color and shape of carapace), the data at this point, do not indicate that morphological differences observed among the groups are statistically significant. More data are needed to evaluate such traits, and any speculation about the genetic architecture of the various traits and coadaptation is premature. Assigning turtles as hybrids based on morphology alone is not prudent at this time. Therefore, different conservation standards for hybrids would create an imminent threat to the endangered hawksbills, loggerheads and olive ridleys. To add to the conservation importance of hybrids, Stronen and Paquet (2013) argue that we need to incorporate conservation goals for such entities and establish guidelines that incorporate conservation value to hybridization. As has been seen with the example of hybrids wolves in the US (Giese 2005), these are challenging policy decisions, which must not overlook the importance of hybrid ecological and evolutionary functions.

Conclusions and future research

Despite population increases observed in the last decades, the contemporary incidence of hybridization as inferred from hatchlings is similar to the observed indices in the adult population surveyed (Fig. 3). This incidence observed in the hatchlings may be even higher, given that only one marker was analyzed. We showed that multiple species paternity is common, and our estimates probably underestimate multiple paternity in these populations in Brazil. Based on the genotypes observed in the progeny, hybrid males are also fertile.

Our study shows for the first time the effect of hybridization on sea turtle fitness, using hatchling viability as a proxy for fitness. Our results indicate that hybrid hatchlings have similar viability to that of homozygous individuals independent of the dam's genotype. The lack of fitness

consequences, in terms of hatchling viability, might explain the similar hatchling production observed between females with conspecific and heterospecific nests. Thus, reinforcement is not likely to occur, and females may continue to mate with either conspecifics or heterospecifics.

The hybridization phenomena described among the populations of loggerheads, hawksbills and olive ridleys in Bahia deserve thorough and continued research. We are pursuing collaborations with partners, to initiate a comprehensive genome-wide analysis of the hybrids and parental species, to increase our power to distinguish between F_1 and backcross progeny, and to better estimate the incidence of multi-species hybrids. Research is also needed on more life history and ecological parameters for the hybrids, such as survivorship at various life stages, age to maturity, growth rates, foraging distribution and ecology. For example, the morphological differences between the three species of sea turtle, largely in the shape of the head and foraging behavior, and how intermediate phenotypes present in the hybrids may affect feeding ability and fitness. How strong is the selection favoring those head shapes? And how strong is selection against intermediates in nature? What are the key features that dictate foraging behavior? Hybrids would seem to provide an interesting tool to study this phenomenon. Perhaps most importantly, we have established a baseline estimate of hatchling hybridization, and look forward to quantifications of this incidence for years to come.

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Author contributions LSS, KAB, ABB and MLW designed the study with input from MAM. Field work and sampling were conducted by LSS, PBL and RM. Laboratory and data analyses were conducted by LSS, RL and ACP with input from MLW, SFM, TBW, KAB, and ABB. LSS drafted the first version of the manuscript, and all authors contributed to the writing of the final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Florida and Projeto TAMAR-ICMBio at which the studies were conducted. This research was approved by the Institutional Animal Care and Use Committees at the University of Florida (201101985) and conducted under SISBIO permit 28938-3 from the Brazilian Ministry of the Environment. Samples were exported under CITES permit 13BR010456/DF, and were imported into the United States under CITES permits 13US724540/9 (Archie Carr Center for Sea Turtle Research).

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