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Does mating behaviour affect connectivity in marine fishes? Comparative population genetics of two protogynous groupers (Family Serranidae)

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Abstract

Pelagic larval duration (PLD) has been hypothesized to be the primary predictor of connectivity in marine fishes; however, few studies have examined the effects that adult reproductive behaviour may have on realized dispersal. We assessed gene flow (connectivity) by documenting variation in microsatellites and mitochondrial DNA sequences in two protogynous species of groupers, the aggregate spawning red hind, *Epinephelus guttatus*, and the single-male, harem-spawning coney, *Cephalopholis fulva*, to ask whether reproductive strategy affects connectivity. Samples of both species were obtained from waters off three islands (Puerto Rico, St. Thomas and St. Croix) in the Caribbean Sea. Despite the notion that aggregate spawning of red hind may facilitate larval retention, stronger signals of population structure were detected in the harem-spawning coney. Heterogeneity and/or inferred barriers, based on microsatellites, involved St. Croix (red hind and coney) and the west coast of Puerto Rico (coney). Heterogeneity and/or inferred barriers, based on mitochondrial DNA, involved St. Croix (coney only). Genetic divergence in both species was stronger for microsatellites than for mitochondrial DNA, suggesting sex-biased dispersal in both species. Long-term migration rates, based on microsatellites, indicated asymmetric gene flow for both species in the same direction as mean surface currents in the region. Red hind had higher levels of variation in microsatellites and lower levels of variation in mitochondrial DNA. Long-term effective size and effective number of breeders were greater for red hind; estimates of θ_f, a proxy for long-term effective female size, were the same in both species. Patterns of gene flow in both species appear to stem in part from shared aspects of larval and adult biology, local bathymetry and surface current patterns. Differences in connectivity and levels of genetic variation between the species, however, likely stem from differences in behaviour related to reproductive strategy.

Keywords: gene flow, groupers, pelagic larval duration, protogyny, reproductive behaviour

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Introduction

Marine fishes have the potential for connectivity over large geographic scales due to high potential fecundity, prolonged pelagic larval phases and the open nature of marine environments (Waples 1987; Levin 2006). Because meteorological and oceanographic conditions are variable across time, reproductive strategies such as spawning site fidelity and pelagic larval duration (PLD) allow many marine fishes to maximize larval survival over 'average conditions' across years (Norcross & Shaw 1984). Consequently, oceanographic and meteorological events that occur during the larval phase are vitally important to connectivity (realized gene flow) and the apportioning of genetic variation across geographic space (Ward *et al.* 1994; Shaklee & Bentzen 1998). For this reason, a primary focus of comparative studies involving gene flow in marine fishes has been the correlation of realized dispersal (gene flow) to traits

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affecting potential dispersal such as PLD and egg buoyancy (Shulman & Bermingham 1995; Bohonak 1999). These studies generally are framed by assaying the distribution of genetic variants in taxonomically related species that have different dispersal potentials across similar geographic ranges (Doherty *et al.* 1995).

Attempts to correlate PLD with estimates of gene flow are somewhat ambiguous, as a strong relationship between the two is reported in some studies (Lacson 1992; Bradbury *et al.* 2008), but not in others (Bowen *et al.* 2006; Weersing & Toonen 2009). One factor complicating a relationship between PLD and connectivity is that larvae may not necessarily behave as passive particles but rather can swim actively and maintain their relative position by taking advantage of local current regimes through both vertical and horizontal movements (Paris & Cowen 2004). It has also been found that localized recruitment may be common in marine fishes (Jones *et al.* 1999; Taylor & Hellberg 2003; Almany *et al.* 2007) and can be affected by factors such as spawning-site location (Swearer *et al.* 1999). The extent to which PLD is correlated to connectivity thus appears to depend on local oceanographic regimes, attributes of larval biology and aspects of adult reproductive behaviour that facilitate local recruitment (Mora & Sale 2002).

One approach to investigating mechanisms generating patterns of connectivity in marine fishes is to examine gene flow between phylogenetically related species with similar PLD but which differ in aspects of reproductive behaviour that would be predicted to affect larval retention. One such behaviour is transient aggregate spawning (Paris *et al.* 2005), the seasonal migration of individuals to specific geographic localities for short, intense bouts of spawning (Sadovy & Domeier 2005). These spawning bouts may be coupled to short-term oceanographic conditions such as slack currents which enhance both fertilization success and larval retention (Whaylen *et al.* 2006; Cherubin *et al.* 2011). For aggregate spawning species, larval retention may be selectively advantageous, as it ensures that larvae and juveniles have access to needed resources, and when mature, access to mates (Choat 2011). If aggregate spawning does enhance localized larval retention, then species with this life history may show reduced connectivity and greater spatial genetic heterogeneity than nonaggregate spawners (Zatcoff *et al.* 2004; Carson *et al.* 2011).

Epinepheline groupers are ideal fishes to compare the effect(s) that reproductive behaviour may have on connectivity and the partitioning of genetic variation. Several species differ in reproductive mode (e.g. aggregate spawning to harem spawning), and many species are protogynous hermaphrodites (Shapiro 1987). The latter often leads to skewed sex ratios which lower effective population size, consequently increasing susceptibility to genetic drift and the rate at which spatial genetic heterogeneity arises (Chopelet *et al.* 2009). Finally, adults of most epinepheline groupers are demersal and reef-associated and exhibit some level of territoriality (Coleman *et al.* 2000).

In this study, we compared patterns of connectivity among island localities in the north-eastern Caribbean Sea between red hind, *Epinephelus guttatus* (Linnaeus, 1758), and coney, *Cephalopholis fulva* (Linnaeus, 1758), two small protogynous groupers that differ in the aspects of reproductive behaviour. Red hind form transient spawning aggregations at specific localities, and spawning occurs for about a week just before the full moon in January (and occasionally in February) when local currents slacken and often change direction, potentially enhancing larval retention (Sadovy *et al.* 1994; Nemeth *et al.* 2008; Cherubin *et al.* 2011). Coney, alternatively, spawn in more spatially diffuse, single-male harems over longer periods of time such that larvae are exposed to temporally averaged current conditions (Sadovy *et al.* 1994; Araujo & Martins 2006). Given grouper PLDs of 30–50 days (Lindeman *et al.* 2001) and the prevailing current patterns in the region (Roberts 1997), larvae of either species originating off one of the islands could be a source of recruits to any of the other islands. These two species thus offer an appropriate comparison for testing the hypothesis that aggregate spawners will have less realized dispersal and will be more genetically heterogeneous across geographic space than nonaggregate spawners, despite similarities in PLD. This comparison is facilitated by the fact that both species are found in shallow coral reef shelf habitats off the islands of St. Croix, St. Thomas and Puerto Rico (Heemstra & Randall 1993), areas with well-described oceanography (Roberts 1997).

Materials and Methods

A total of 416 red hind and 397 coney were sampled between 2008 and 2010 from offshore localities near St. Croix (STC), St. Thomas (STT) and the east and west coasts of Puerto Rico (PRE and PRW, respectively). Tissues (fin clips) were obtained from commercial fish houses or directly from artisanal fishers and were stored in 95% nondenatured ethanol or 10% DMSO buffer (Seutin *et al.* 1991). DNA was extracted following a modified Chelex extraction protocol (Estoup *et al.* 1996). After a final 2-min centrifugation at 13 000 ***g***, 1–2 μL of supernatant was used as a template for subsequent PCR reactions.

A total of 19 microsatellites for red hind and 24 for coney were assayed; of these, 12 microsatellites were assayed in common for both species. The forward

primer from each primer pair was labelled with one fluorescent label of Dye Set D (Applied Biosystems): 6-FAM, HEX or NED. Descriptions of primers and protocols of PCR amplifications may be found in Renshaw *et al.* (2010). Amplicons were electrophoresed on 6% polyacrylamide gels, using an ABI Prism 377 sequencer (Applied Biosystems) and the GeneScan 400HD ROX Size Standard (Applied Biosystems) in each lane. Scoring was conducted manually, using GENESCAN v.3.1.2 (Applied Biosystems) and GENOTYPER v.2.5 (Perkin Elmer).

A fragment of the mitochondrially encoded NADH-dehydrogenase subunit 4 (ND4) was sequenced for both species, using primer pairs Egu_275F (5′-TCCCACCTTAATGCTTGTCC-3′) and Egu_851R (5′-ATAACTTGTCGGCGTTGGAT-3′) for red hind and Cfu_338F (5′-GAACCGATCGTCTTCAAAGC-3′) and Cfu_1176R (5′-CCCACAGTGCGAAGATTAAGA-3′) for coney. Thirty-microlitre PCR contained 1× reaction buffer (pH 8.5), 2 mM $MgCl_2$, 0.25 mM of each dNTP, 15 pmol of each primer, 0.1 U/μL *Taq* polymerase and 2 μL of template. Reaction conditions consisted of an initial denaturation at 95 °C for 4 min followed by 45 cycles of 95 °C for 1 min, 62 °C (red hind) or 64 °C (coney) for 45 s and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. Amplified products were column-cleaned using QIAquick® PCR purification kits (Qiagen) and then sequenced in the forward and reverse direction, using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Warrington, UK). Five-microlitre sequencing reactions consisted of 10–40 ng of template, 0.25 μL of BigDye master mix, 0.875 μL of BigDye 5× reaction buffer and 32 pmol of F or R primer. Sequencing conditions consisted of denaturation at 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Amplifications were electrophoresed on an ABI 3100 Sequencer (Applied Biosystems Inc.) through 50-cm capillaries. Sequence chromatograms were edited in SEQUENCHER v.4.8 (Gene Codes Corporation), resulting in a 500-bp fragment for red hind and a 670-bp fragment for coney; the ND4 sequence from red hind was contained entirely within the fragment sequenced for coney.

Conformance to the expectations of Hardy–Weinberg equilibrium (HWE) was evaluated for each microsatellite in each sample, using GENEPOP v.4.0 (Raymond & Rousset 1995; Rousset 2008); significance was assessed at the 0.05 level, using exact tests with 1000 batches and 10 000 iterations per batch. Sequential Bonferroni adjustment (Rice 1989) was used to correct for multiple testing. MICROCHECKER v.2.2.3 (van Oosterhout *et al.* 2004) was used to screen for null alleles and genotyping error. Number of alleles, allelic richness and unbiased gene diversity (expected heterozygosity) were estimated for each microsatellite in each geographic sample for each species, using FSTAT v.2.9.3.2 (Goudet 2001). Wilcoxon signed rank tests, implemented in SYSTAT 8.0 (SPSS Inc.), were used to test for homogeneity in allelic richness and gene diversity between pairs of geographic samples within each species. Signed rank ANOVAs, implemented in SYSTAT, were used to test homogeneity of allelic richness and gene diversity across species and localities, using only the 12 microsatellites assayed in both species.

Homogeneity in allele and genotype distributions (microsatellites) among sample localities in each species was tested using single-level analysis of molecular variance (AMOVA), as implemented in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010). Pairwise F_{ST} values (between samples in each species), based on microsatellites, also were estimated using ARLEQUIN. Significance of pairwise F_{ST} values at the 0.05 level was assessed by permuting individuals between samples 10 000 times. Correction for multiple testing was implemented using the false discovery rate (FDR) procedure of Benjamini & Hochberg (1995). LOSITAN (Beaumont & Nichols 1996; Antao *et al.* 2008) was used to screen for F_{ST} outliers (candidate loci under selection) by comparing observed mean F_{ST} values at each microsatellite, corrected for locus-specific gene diversity (expected heterozygosity), against a 95% confidence interval of F_{ST} values (corrected for diversity) generated by simulation. The simulation was implemented with 20 000 steps and a FDR of 0.01. Simulation indicated a single outlier microsatellite in each species; sequences of these two microsatellites were screened using the BLASTN algorithm within NCBI's BLAST suite (Altschul *et al.* 1990) to identify putative function. Pairwise F_{ST} estimates, excluding the outlier loci, were then generated to assess whether the outlier microsatellites had a detectable effect on the initial estimates of F_{ST}. Correspondence analysis (Guinand 1996) as implemented in GENETIX v.4.05.2 (Belkhir *et al.* 2004) was used to visualize the relationship of individual microsatellite genotypes within and among samples in each species.

Nucleon diversity (h) and nucleotide diversity (π) were calculated for mitochondrial ND4 sequences at each sample location in each species, using ARLEQUIN. Friedman's rank test, implemented in SYSTAT, was used to test homogeneity of h and π between the two species. Homogeneity of haplotype (mtDNA) distributions among sample localities in each species was tested using single-level analysis of molecular variance (AMOVA), as implemented in ARLEQUIN. Distances were calculated using a Tamura–Nei (TrN) model (Tamura & Nei 1993), with a gamma parameter of 0.011, as selected by JMODELTEST v.0.1.1 (Guindon & Gascuel 2003; Posada 2008). Pairwise Φ_{ST} values were estimated using

ARLEQUIN, with significance determined as described previously for microsatellites. Mantel tests (Smouse *et al.* 1986) were implemented in ARLEQUIN (100 000 permutations) to evaluate whether pairwise F_{ST} (microsatellites) and Φ_{ST} (ND4 sequences) estimates were correlated.

Patterns of average, long-term connectivity (migration rate, m) between geographic samples in each species and average, long-term effective size (N_{eLT}) in each geographic sample (both species) were estimated using the microsatellite data and the Bayesian approach implemented in MIGRATE v.3.2.16 (Beerli & Felsenstein 2001; Beerli 2006). For red hind, both panmixia and full migration models were used initially, as tests of spatial genetic homogeneity among the geographic samples of red hind were equivocal. Replicates for both models were run using a burn-in period of 15 000 steps, followed by 7 000 000 steps, with trees recorded every 100 steps; a total of 70 000 trees were sampled. A comparison of marginal likelihoods and subsequent estimation of the probability of both models (Beerli & Palczewski 2010) revealed that it was unlikely ($P < 0.0001$) that the samples of red hind came from a single panmictic population; hence, only results from the full migration model are reported. For coney, where tests of spatial genetic homogeneity among geographic samples were unequivocal, replicate runs used the full migration model, with four connected chains with static heating (temperatures 1.0, 1.5, 3.0 and 100 000) and a burn-in period of 150 000 steps, followed by 10 000 000 steps with trees recorded every 100 steps; a total of 100 000 trees were sampled. For both species, asymmetry of overall gene flow was examined for each pairwise set of m estimates by comparing overlap of confidence intervals. To obtain estimates of m (where $m = M/\mu$) and N_{eLT}, (where $\theta = 4N_e\mu$), the modal mutation rate (μ) of the microsatellite data set was obtained using the Bayesian coalescent approach of Beaumont (1999) and Storz & Beaumont (2002) as implemented in MSVAR v. 1.3. BOA (Smith 2005) was used to estimate the 95% highest posterior density interval for each point estimate of μ.

Effective number of breeders (N_b) was estimated for each geographic sample in each species, using the linkage disequilibrium approach in LDNE (Waples 2006; Waples & Do 2008). Minor alleles at a frequency of 0.02 or less were excluded, following Portnoy *et al.* (2009); confidence intervals were obtained using jackknifing. A coalescent-based, maximum-likelihood algorithm, as implemented in FLUCTUATE (Kuhner *et al.* 1998), was used to estimate θ_f (where $\theta_f = 2N_{ef}\mu$) for both species at each locality, using a homologous 500 bp of ND4 sequence data. Given the difficulties inherent in estimating μ for mitochondrial DNA data, and the assumption that μ is equivalent in the two species, θ_f was taken as a proxy for average, long-term female effective size (N_{ef}) and compared directly. Replicate runs consisted of 50 short chains with 25 000 steps followed by 25 long chains of 250 000 steps. Initial starting values for θ_f were based on Watterson's (1975) estimator.

The geographic orientation of primary and secondary barriers to gene flow among sample localities in each species was visualized using BARRIER v.2.2 (Manni *et al.* 2004). Divergence between pairs of localities (microsatellites, both species) employed chord distances (Cavalli-Sforza & Edwards 1967), estimated with MSA v.4.05 (Dieringer & Schlötterer 2003); divergence of ND4 sequences (coney only) employed TrN distance, estimated with MEGA v.4.0 (Tamura *et al.* 2007) with a gamma parameter of 0.011. BARRIER analysis, using ND4 sequences of red hind, was not carried out as none of the tests of spatial homogeneity of red hind mtDNA haplotypes were significant. BARRIER was run iteratively to define primary and secondary barriers; significance was assessed by bootstrapping over microsatellites, using MSA (Dieringer & Schlötterer 2003), and over mtDNA haplotypes, using a PERL script, within geographic samples and recalculating distances 1000 times.

Results

Summary statistics for microsatellites are presented in Table S1 (Supporting information). Genotypes at all microsatellites assayed in both species may be found at http://agrilife.org/gold/doc/ and DRYAD (doi: 10.5061/dryad.sj894) under the file name 'Microsatellite genotypes of red hind and coney'. Genotypes at nine microsatellites (four in red hind, five in coney) deviated significantly from the expectations of HWE prior to Bonferroni correction; following correction, only genotypes at RH-GATA-15 in red hind from PRW deviated significantly from the expectations of HWE. Analysis with MICROCHECKER indicated the deviation at RH-GATA-15 could be due to null alleles. Subsequent analyses (in red hind) were executed with and without RH-GATA-15; virtually, no differences were found, so all subsequent analyses include this microsatellite. The average number of alleles (19 microsatellites) assayed per locality (±SE) for red hind ranged from 14.5 ± 2.3 (PRE) to 15.3 ± 2.3 (STC); for coney (24 microsatellites), the average (±SE) ranged from 9.3 ± 1.5 (STT) to 9.9 ± 1.6 (STC). Allelic richness and gene diversity (expected heterozygosity) followed the same pattern in each species. Pairwise tests in each species of homogeneity in allelic richness and gene diversity were nonsignificant. For the 12 microsatellites that were amplified in both species, allelic richness and gene diversity were greater in red hind (12.7 vs. 8.5 and 0.582 vs. 0.494, respectively). Signed rank ANOVA revealed that a signifi-

cant component of the variance in allelic richness ($P = 0.031$) but not gene diversity ($P = 0.077$) was attributable to species. Summary statistics for mtDNA haplotypes also are presented in Table S1 (Supporting information); the distribution of individual haplotypes and their GenBank accession numbers are presented in Table S2 (Supporting information). A total of 21 different haplotypes were found in red hind, while 40 different haplotypes were found in coney. Estimates of h and π in red hind ranged from 0.757 (STT) to 0.882 (STC) and from 0.0031 (STT) to 0.0040 (STC), respectively; estimates of h and π in coney ranged from 0.893 (PRW) to 0.920 (STC) and from 0.0032 (PRW) to 0.0042 (STC), respectively. Nucleon diversity (h) differed significantly between the two species ($Q = 4.0$, d.f. = 1, $P = 0.046$), whereas nucleotide diversity (π) did not ($Q = 1.0$, d. f. = 1, $P = 0.317$).

Analysis of molecular variance (AMOVA), based on microsatellites, revealed marginally significant heterogeneity ($\Phi_{ST} = 0.0006$, $P = 0.050$) among samples of red hind and highly significant heterogeneity ($\Phi_{ST} = 0.0023$, $P < 0.0001$) among samples of coney (Table 1). Estimates of F_{ST} (and tests of the null hypothesis $F_{ST} = 0$) between pairs of samples of red hind were significant before correction for STT vs. STC and STC vs. PRW; only the former remained significant after correction (Table 2). One microsatellite, *Cfu*9, was identified as an outlier ($F_{ST} = 0.029$, $P = 0.0096$). A BLASTN search identified a region of high similarity (90%, E-value = $1e^{-31}$) between the original cloned sequence and a miscellaneous RNA putatively identified as a helicase-DNA-binding protein in *Oreochromis niloticus*. When pairwise estimates of F_{ST} were generated with *Cfu*9 omitted, the F_{ST} of STT vs. STC was significant before but not after correction, and the F_{ST} of STC vs. PRW was no longer significant. Estimates of F_{ST} between pairs of samples of coney were significant after correction for STT vs. STC and for all comparisons involving PRW (Table 2). One microsatellite, *Cfu*10, was identified as an outlier ($F_{ST} = 0.079$, $P < 0.001$). A BLASTN search failed to identify any highly similar contiguous regions. When pairwise estimates of F_{ST} were generated with *Cfu*10 omitted, F_{ST} values in comparisons involving PRW decreased slightly but all remained significant after correction as did the comparison of STC vs. STT. Correspondence analysis revealed considerable overlap in microsatellite genotypes of all four samples of red hind (Fig. 1a), although the data points did not appear to be completely admixed. Correspondence analysis, using all four samples of coney, indicated that PRW was distinct from the other locations (Fig. 1b); when PRW was excluded, the remaining three samples appeared more distinct from one another (Fig 1c).

AMOVA, based on mtDNA haplotypes, was nonsignificant ($\Phi_{ST} = -0.0090$, $P = 0.69$) for red hind, but significant ($\Phi_{ST} = 0.0298$, $P = 0.045$) for coney (Table 1). Pairwise Φ_{ST} values in comparisons involving red hind ranged from −0.0090 to 0.0190 and did not differ significantly from zero (Table 2). Pairwise Φ_{ST} values in comparisons of coney were significant before but not after correction for PRE vs. STC and PRW vs. STC (Table 2). To account for differences in ND4 fragment sizes, ND4 sequences in coney were trimmed to the same (homologous) 500-bp fragment used for red hind. Thirty-one of 40 coney haplo-

Table 1 Results of single-level AMOVA for red hind and coney, based on microsatellite genotypes and mtDNA sequences

d.f., degrees of freedom; SS, sum of squares; VC, variance component; %V, per cent of variance.

	d.f.	SS	VC	%V	Φ_{ST}	*P*-value
Red hind						
Microsatellites						
Among populations	3	22.69	0.0042	0.06	0.00064	0.050
Within populations	790	5203.12	6.5862	99.94		
Total	793	5225.39	6.5904			
mtDNA						
Among populations	3	3.975	−0.0162	−0.9	−0.00897	0.690
Within populations	119	216.74	1.8214	100.9		
Total	122	220.72	1.8052			
Coney						
Microsatellites						
Among populations	3	28.27	0.0145	0.23	0.00227	<0.001
Within populations	828	5300.58	6.4017	99.77		
Total	831	5328.86	6.4162			
mtDNA						
Among populations	3	14.50	0.0761	2.98	0.02982	0.045
Within populations	120	297.30	2.4753	97.02		
Total	123	311.53	2.5514			

Table 2 F_{ST} (microsatellite genotypes, above diagonal) and Φ_{ST} (ND4 sequences, below diagonal) values for pairwise comparisons of sample localities for red hind and coney

	STT	STC	PRE	PRW
Red hind				
STT	—	**0.0019***	0.0006	−0.0009
STC	−0.0085	—	0.0007	**0.0013**
PRE	0.0190	−0.0090	—	0.0002
PRW	0.0047	−0.0082	−0.0053	—
Coney				
STT	—	**0.0017***	−0.0004	**0.0050***
STC	0.0250	—	0.0004	**0.0030***
PRE	0.0130	**0.0520**	—	**0.0019***
PRW	0.0015	**0.0710**	0.0084	—

Values significant at $\alpha = 0.05$ before correction are in boldface. Values significant after correction are denoted with an asterisk.

types were recovered, and patterns of mtDNA homogeneity inferred were essentially identical to those when all ND4 sequences were used. Mantel tests were nonsignificant, indicating that pairwise F_{ST} and Φ_{ST} values were not correlated in either species ($P = 0.832$, red hind; $P = 0.250$, coney).

The average mutation rate (μ) across loci, obtained from MSVAR, was 2.7×10^{-4} (CI: 9.3×10^{-5} and 7.8×10^{-4}) and 3.0×10^{-4} (CI: 1.0×10^{-4} and 8.1×10^{-4}) for red hind and coney, respectively. Estimates of m (average, long-term migration rate) among samples of red hind ranged from 0.12% (PRW to PRE) to 0.21% (STC to PRE); confidence intervals of m overlapped for all comparisons except for STC vs. PRE, where larger values of m were associated with east-to-west gene flow (Table 3). Estimates of m among coney ranged from 0.18% (STT to STC) to 0.30% (STT to PRW). For coney, confidence intervals of m for two comparisons (STT vs. PRE and STT vs. PRW) did not overlap; all asymmetries were associated with east-to-west gene flow (Table 3).

Estimates of average, long-term effective population size (N_{eLT}), effective number of breeders (N_b) and a proxy for average, long-term effective female population size (θ_f) for each species are shown in Table 4. Estimates of N_{eLT} for red hind by locality ranged from 3673.6 (PRE) to 4212.3 (STC) but did not differ significantly from one another; N_{eLT} estimates for coney by locality ranged from 2160.6 (PRE) to 2651.8 (STC) and also did not differ significantly from one another. On average, estimates of N_{eLT} in coney were approximately 42% lower than those in red hind. The N_b estimate for red hind at STT was infinity; for the remaining samples of red hind, N_b estimates ranged from 1083.5 (STC) to 2944.1 (PRE) and did not differ significantly from one another. Estimates of N_b for coney ranged from 465.3 (STC) to 776.5 (PRE); none of the point estimates of N_b for coney differed significantly from one another. On average, estimates of N_b in coney were approximately 37% lower than for red hind. The largest point estimate of N_b for both species was for PRE, while the smallest in both species was for STC. Estimates of θ_f for red hind ranged from 0.0046 (PRW) to 0.0095 (STC) but did not differ significantly from one another. Estimates of θ_f for coney ranged from 0.0059 (STT) to 0.0128 (STC) and also did not differ significantly from one another.

Bootstrap resampling of inferred barriers, based on microsatellites, indicated restricted gene flow in red hind between STC and STT (primary barrier, 55.4% support; secondary barrier, 37.3% support) and between PRW and the other three localities (primary barrier, 44.6% support; secondary barrier, 54.4% support). Inferred barriers, based on microsatellites, also indicated restricted gene flow in coney (Fig. 2a). One separated PRW from the other localities (primary barrier, 87.4% support; secondary barrier, 12.6% support), while a set of barriers indicated restricted gene flow between STC and PRE (primary barrier, 11.6% support; secondary barrier, 71.4% support) and STC and STT (primary barrier, 12.6% support; secondary barrier, 66.2% support). Inferred barriers based on ND4 sequences (coney only, Fig. 2b) identified restricted female gene flow between STC and PRE (primary barrier, 87.8% support; secondary barrier, 10.9% support), STC and STT (primary barrier, 88.8% support; secondary barrier, 6.2% support) and STT and PRE (primary barrier, 21.6% support; secondary barrier, 55.7% support). The barrier isolating PRW from the other localities, inferred based on microsatellites, received little statistical support from ND4 sequences (Fig. 2b).

Discussion

In this study, we asked whether differences in reproductive behaviour can lead to differences in realized dispersal (connectivity) in marine fishes. We compared patterns of genetic variation and divergence at the same four localities between two species of epinepheline groupers that share several characteristics hypothesized to affect connectivity. These characteristics include pelagic larval duration or PLD (Lindeman *et al.* 2001), basic life history and habitat preferences (Bolden 1994); both species also are subject to the same oceanic circulation patterns. The two species are protogynous hermaphrodites but have a major difference in reproductive strategy. Red hind form transient spawning aggregations at specific localities and spawn over a relatively short period of time when oceanographic conditions are thought to enhance larval retention (Sadovy *et al.* 1994; Nemeth *et al.* 2008; Cherubin *et al.* 2011); coney,

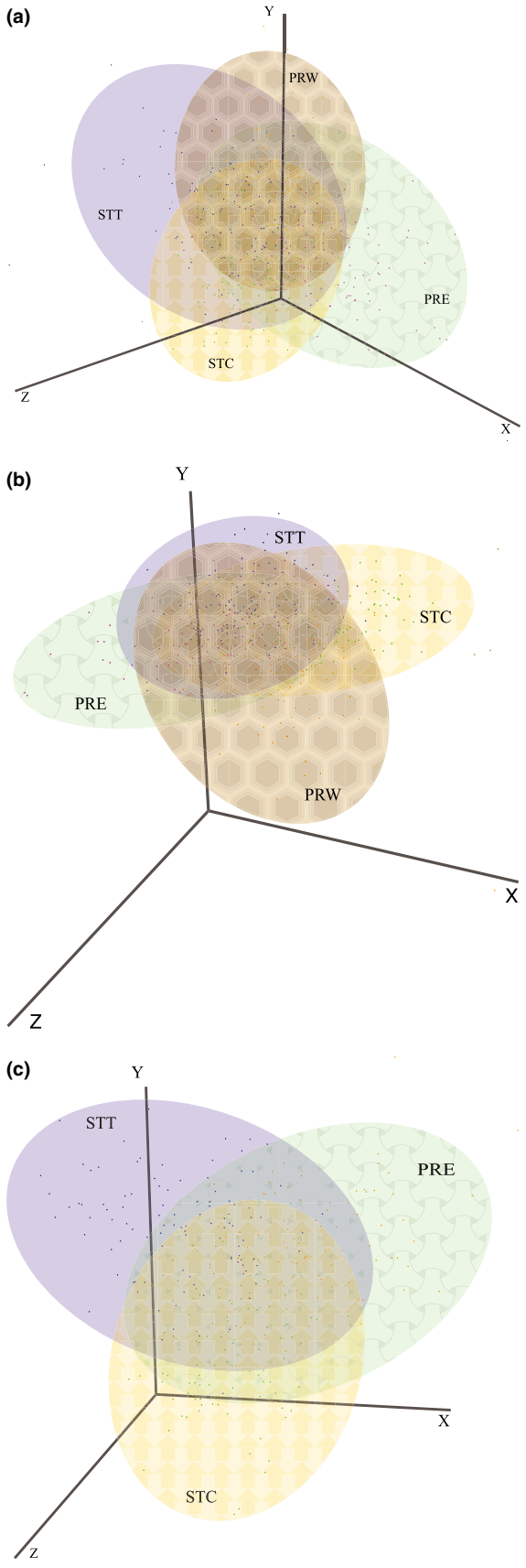

Fig. 1 Correspondence analysis, using microsatellite genotypes, for (a) all four localities of red hind, (b) all four localities of coney and (c) three localities (PRW excluded) of coney. STT, St Thomas; STC, St. Croix; PRE, Puerto Rico East; and PRW, Puerto Rico West. Ellipses surrounding points include 90% of samples for a give locality. Figure created using the R Commander package for R (Fox 2005).

alternatively, spawn in more spatially diffuse, single-male harems over longer periods of time (Sadovy *et al.* 1994; Araujo & Martins 2006). We found differences between the two species in spatial genetic heterogeneity and patterns (magnitude and variability) of genetic variation across the area sampled. However, contrary to expectation, red hind have limited population structure, with higher levels of allelic richness (microsatellites) and lower levels of nucleon diversity (mitochondrial DNA), whereas coney have more extensive population structure, with lower allelic richness and higher nucleon diversity. Estimates of both average, long-term effective population size and effective number of breeders also were greater in red hind; estimates of θ_f, a proxy for average, long-term effective female population size, however, were essentially the same in both species. Finally, in both species, divergence in microsatellites across localities was greater than divergence in mitochondrial DNA. This and the absence of a correlation in both species between divergence in microsatellites and mitochondrial DNA suggests that dispersal in both species is likely sex-biased, with either greater dispersal in females, philopatric behaviour in males or both.

The genetic heterogeneity and/or barriers to gene flow, based on microsatellites, detected between St. Croix (STC) and the other three localities likely results from several common factors that potentially could impede gene flow. First, STC is separated from the other sites by an open ocean expanse that features a deep trench extending to 4200 m (Rogers *et al.* 2008), which likely restricts adult dispersal. Second, although epinepheline groupers have fairly long PLD (Lindeman *et al.* 2001; Marancik *et al.* 2012), their larvae are primarily found in near-shore shelf water and do not appear to disperse readily offshore (Leis 1987). Third, the sample from STC was obtained on the leeward side of the island, and there is evidence that leeward island sites are prone to larval retention (Swearer *et al.* 1999). Finally, surface currents in the region are almost all to the west (Roberts 1997), including through the Anegada Passage, a fairly wide channel that connects the Atlantic Ocean with the Caribbean Sea and runs westward between STC and STT and to the south of Puerto Rico (Johns *et al.* 2002). The relative isolation of STC from the other sites sampled also has been detected in lutjanine snappers (Carson *et al.* 2011; Saillant *et al.* 2012),

Table 3 Point estimates of *m* (migration rate), based on microsatellite genotypes, for pairwise comparisons of samples of red hind and coney from St. Thomas (STT), St Croix (STC), the west coast of Puerto Rico (PRW) and the east coast of Puerto Rico (PRE)

	STT	STC	PRE	PRW
Red hind				
STT	—	0.189	0.179	0.168
STC	0.181	—	**0.206**	0.126
PRE	0.191	**0.139**	—	0.140
PRW	0.169	0.161	0.123	—
Coney				
STT	—	0.176	**0.281**	**0.299**
STC	0.221	—	0.257	0.250
PRE	**0.200**	0.217	—	0.235
PRW	**0.199**	0.208	0.202	—

Row entries are donor populations; column entries are recipient populations. Estimates in boldface are those with nonoverlapping 95% confidence intervals.

suggesting that these common factors may disrupt gene flow in a variety of reef-associated species.

The genetic heterogeneity and/or barriers to gene flow, based on microsatellites, detected between the west coast of Puerto Rico (PRW) and the other three localities, also could be due in part to larval retention and bathymetry. The west coast is the leeward side of the island, and passage of either larvae or adults along the northern coast seems unlikely given its high wave energy, high fluvial effluent and absence of shallow coral-reef habitat (Schneidermann *et al.* 1976). There also is a west-to-east, near-shore counter current along the southern shelf of Puerto Rico (Roberts 1997) that could disrupt larval connectivity between PRW and the other localities. However, divergence (measured as pairwise F_{ST}) and inferred barriers to gene flow, based on microsatellites, between PRW and the other localities were far stronger in coney than in red hind. Because of similarities in both species in factors associated with connectivity (i.e. PLD, current patterns, general larval retention mechanisms, bathymetry), the strong difference in the degree of microsatellite divergence indicates that there are additional factors affecting dispersal that differ between the species and limit connectivity in coney far more than in red hind.

The differences between the two species in realized connectivity, patterns of microsatellite variation and microsatellite-based estimates of both long-term effective size and effective numbers of breeders are best accounted for by the differences between the species in mating behaviour and dispersal potential, particularly in males. Haremic male coney maintain breeding territories in shallow shelf coral reef habitats for periods of time as long as 10 months (Colin *et al.* 1987; Sadovy *et al.* 1994; Araujo & Martins 2006). Resident males may occupy the same territory for multiple years, given the assumed 25-year lifespan and a transition age of 3 years (Araujo & Martins 2006). Aggregate-spawning groupers, alternatively, are known to move considerable distances (200–1000 km) from spawning sites (Bolden 2000; McGovern *et al.* 2005), and red hind tagged and released at spawning sites off STC and STT have been recaptured off the islands of Culebra and Vieques along the eastern coast of Puerto Rico (Nemeth *et al.* 2007). Finally, although male red hind tend to remain near spawning sites between bouts of spawning while females depart (Nemeth *et al.* 2007), males occupy deeper shelf habitats at other times (Shapiro *et al.* 1994) and potentially can join other aggregations in subsequent years. Taken together, these observations and the results obtained from this study suggest greater male philopatry in coney. It also is possible that fewer male–female pairings in coney may contribute, on average, to subsequent generations, given that the number of male–female

Table 4 Estimates of average, long-term effective population size (N_{eLT}), effective number of breeders (N_b) and θ_f (a proxy for average, long-term effective female population size) with lower (0.025) and upper bounds (0.975) of 95% confidence intervals. N_{eLT} and N_b are based on microsatellites; θ_f is based on mitochondrial DNA

	0.025	N_{eLT}	0.975	0.025	N_b	0.975	0.025	θ_f	0.975
Red hind									
STT	1178.7	4063.7	13367.2	—	∞	—	0.0039	0.0084	0.0164
STC	1188.4	4212.3	13822.6	600.4	1083.5	4813.2	0.0048	0.0095	0.0181
PRE	1082.1	3673.6	13206.5	971.0	2944.1	∞	0.0036	0.0078	0.0159
PRW	969.4	3933.6	12135.0	655.3	1254.1	10643.2	0.0020	0.0046	0.0091
Coney									
STT	813.0	2188.2	7687.6	366.1	673.2	3159.0	0.0028	0.0059	0.0123
STC	985.2	2651.8	9544.4	306.8	465.3	908.1	0.0057	0.0128	0.0240
PRE	695.3	2160.6	7573.6	420.7	776.5	3914.2	0.0034	0.0072	0.0141
PRW	684.9	2204.8	8094.8	303.6	575.3	3400.3	0.0029	0.0077	0.0143

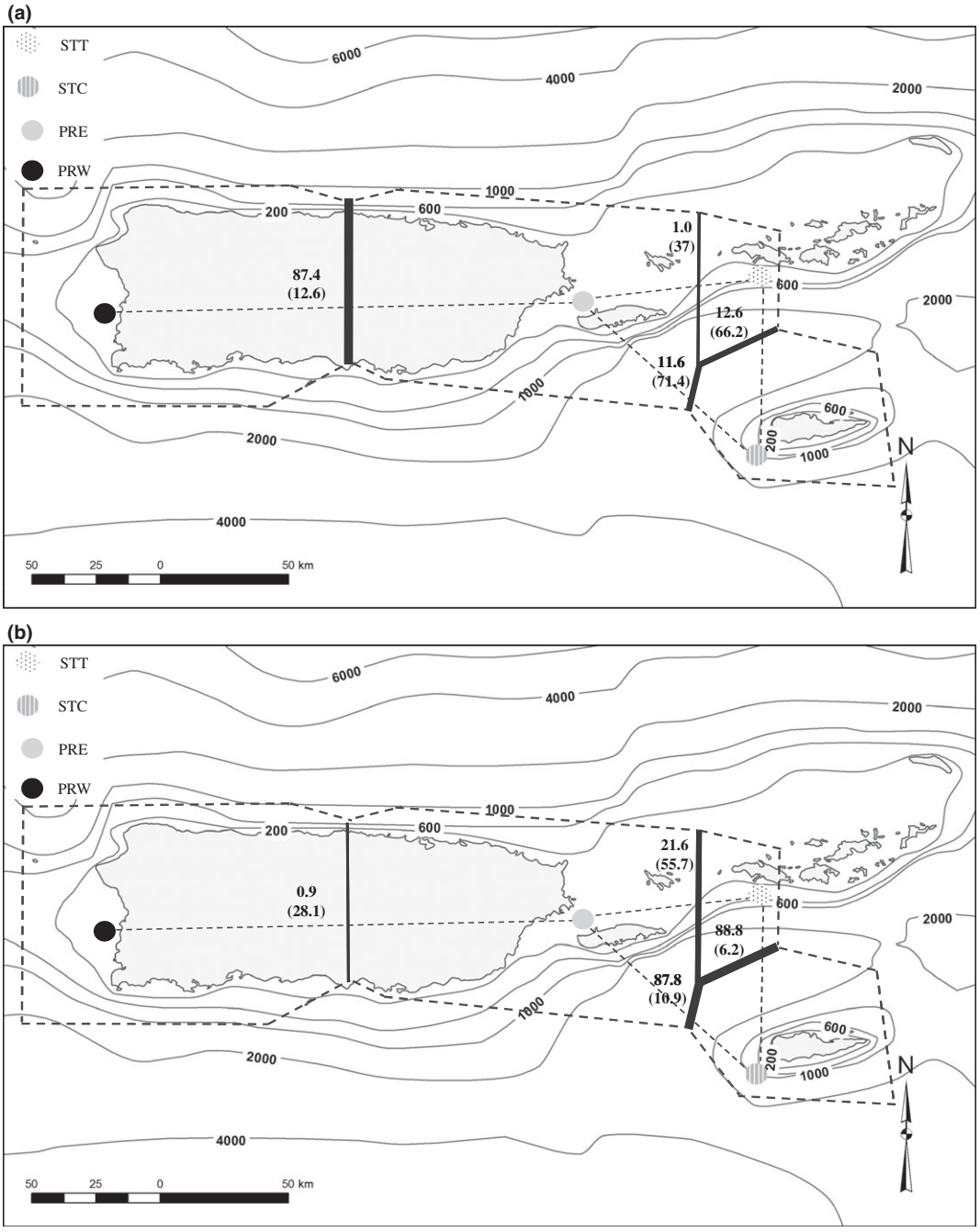

Fig. 2 Voronoi diagram (dashed lines) and inferred barriers to gene flow: (a) coney, microsatellites; and (b) coney, ND4 sequences. Edges separating nearest neighbour localities are denoted by solid lines; thickness of lines is proportional to the number of times an edge was identified as a primary or secondary barrier, based on 1000 bootstrapped data sets. Upper number is the percentage of time edge was identified as the primary barrier; lower number (in parentheses) is the number of time edge was identified as secondary barrier.

pairings is related directly to effective size (Crow & Kimura 1970), and both N_{eLT} and N_b were on average >35% smaller for coney than for red hind.

Except for comparisons with STC, the absence of both heterogeneity and strong barriers to gene flow, based on mtDNA, indicates that females in both species may be largely responsible for maintaining connectivity among localities. This may be due in part to the fact that all monandric, protogynous hermaphrodites begin life as females, meaning that all settling juveniles are

female. In addition, females, unlike males, are unrestricted by the need to defend breeding territories and may move across male territories to assess mate quality (Warner 1987) or to establish territories as they near impending sex change and transition to males (Robertson 1972; Raihani *et al.* 2012). In red hind, for example, females spawn repeatedly and potentially visit more than one aggregating site in the same year (Sadovy *et al.* 1994); there also is evidence supporting a relationship between size and migratory distance that is positive for females and negative for males (Nemeth *et al.* 2007). However, pairwise tests of homogeneity and analysis using BARRIER, based on mitochondrial DNA, indicated restricted gene flow between STC and both PRE and PRW in coney but not in red hind. The difference between the species could reflect greater female-mediated gene flow in red hind. This is somewhat difficult to believe as the depth preference of red hind extends from 2 to 100 m (Heemstra & Randall 1993), which should preclude easy passage across the 4200-m trench (Rogers *et al.* 2008) separating STC from the other localities.

The absence of genetic heterogeneity or barriers to gene flow, based on both microsatellites and mitochondrial DNA, between STT and PRE suggests that the east-to-west surface currents which dominate the region (Roberts 1997) readily disperse larvae to PRE from STT. This is supported by the observation that migration asymmetries between these two localities were significant and in an east-to-west direction. We also found significant migration asymmetry, based on microsatellites, in an east-to-west direction between STC and PRE in red hind. This is noteworthy given the pairwise tests and analysis with BARRIER (above) which indicated female connectivity between STC and PRE. Also, estimates of N_b for both species were larger for samples from PRE, which could be caused by input of larvae from STT and STC.

The significantly lower haplotype diversity in red hind is possibly related to overexploitation of this species in the region over the past three decades (Beets *et al.* 1994; Matos-Caraballo 2004). Typically, female red hind outnumber males by a ratio of approximately four to one; by the late 1980s, however, the ratio of females to males in the region was skewed to as high as 15 to one (Beets & Friedlander 1999). A high skew in sex ratio can indicate sperm limitation (Beets & Friedlander 1999) which could limit the overall number of contributing females, theoretically reducing both haplotype variation and effective number of females. While we did not find a difference between the two species in θ_f, the confidence intervals estimated for θ_f are not especially exclusive given that mitochondrial DNA represents only a single genetic locus.

This study demonstrates that aspects of adult behaviour are critical elements in determining levels of connectivity and partitioning of genetic variance in marine fishes. For protogynous groupers, which typically are long lived, territorial, top-level predators with relatively small census sizes and carrying capacities limited by the availability of specific habitat (Leis 1987; Coleman *et al.* 2000; Levin & Grimes 2002), movement of reproductively active adults may be especially important to gene flow if density dependence limits entry of recruited juveniles into the breeding population.

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D.S.P. had responsibility for data collection, analysis and for writing the manuscript. C.M.H. and M.A.R. were involved in data collection and contributed to data analysis and writing. N.J.C. had responsibility for coordinating sample collection in the field and contributed to writing. J.R.G. had responsibility for data analysis, writing and for sample collection.

Data accessibility

Genotypes at all microsatellites assayed in both species may be found at http://agrilife.org/gold/doc/ and DRYAD (doi: 10.5061/dryad.sj894) under the file name 'Microsatellite genotypes of red hind and coney.'

DNA sequences: GenBank Accession nos for red hind JX402700–JX402720 and coney JX402660–JX402699.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Summary statistics for microsatellite and mtDNA haplotype data.

Table S2 Distribution of individual haplotypes and their GenBank Accession nos.