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ORIGINAL ARTICLE

Contact zone dynamics during early stages of speciation in a chorus frog (*Pseudacris crucifer*)

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Characterizing the genetic and behavioural consequences of contact between previously geographically isolated lineages provides insights into the mechanisms underlying diversification and ultimately speciation. The spring peeper (*Pseudacris crucifer*) is a widespread Nearctic chorus frog with six divergent mitochondrial DNA (mtDNA) lineages, many of which came into secondary contact during the Holocene. We examined genetics, morphology, advertisement calls and female preference for two lineages that began diverging in allopatry in the Pliocene and now overlap in southwestern Ontario, Canada. We found non-coincident clines in mtDNA and nuclear DNA, mirroring directionality of premating isolation barriers. We also found divergence in a range of traits between these two lineages, displacement in male call attributes and female preference for calls of their natal lineage in sympatry. Hybrids were morphologically distinct from both parental lineages, but hybrid male calls were acoustically intermediate. Female hybrids showed asymmetrical preference for Eastern male calls. These results considered together provide evidence of either unidirectional hybridization or selection against hybrids, potentially implying reproductive character displacement. Our work demonstrates the utility of integrated, multi-character approaches to understanding the processes of divergence and the nature of speciation.
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INTRODUCTION

The now substantial phylogeographic literature reveals that deep genealogical divisions are prevalent within many traditionally classified species, irrespective of evolutionary affinity or biogeographic region (Soltis *et al.*, 2006; Bickford *et al.*, 2007). What is less clear is whether such cryptic lineages are nascent or fully independent species, or whether they simply represent 'evolutionary ephemera' (Avise and Wollenberg, 1997), where differences among genetic populations will disappear over time. Although many phylogeographic studies have tacitly assumed the former, this proposition remains untested for most taxa. An integrated, multi-character approach is necessary to understand the processes involved in speciation and to catalogue the true species diversity represented in deeply differentiated widespread taxa (Lougheed *et al.*, 2006).

Contact zones represent natural experiments that help us disentangle the roles of different evolutionary processes in speciation (Harrison, 1993). The outcome of secondary contact between diverging populations may be shaped by many factors such as length of previous isolation in allopatry, mate recognition system, environmental context or population densities (see, for example, Dufresnes *et al.,* 2014). Possible outcomes include hybridization and eventual dissolution of differences, creation of a stable hybrid zone or accentuation of differences in zones of sympatry including ecological and reproductive character displacement (RCD, see Dobzhansky, 1937). The best systems for studying these mechanisms are taxa that either have lineage pairs in secondary contact over broad geographic areas or have secondary contact zones among multiple pairs of evolutionary lineages spanning a range of divergence times (Feder *et al.*, 2013). In those cases, one can potentially separate the relative importance of spatial, ecological and genetic factors in maintaining independence of divergent lineages.

Phylogeographic studies of the spring peeper (*Pseudacris crucifer*), a widespread eastern North American chorus frog, reveal a dynamic history of geographic isolation, range expansion and secondary contact among six divergent mitochondrial DNA (mtDNA) lineages (Figure 1; Austin *et al.,* 2002, 2004). These lineages diverged because of geographic isolation in multiple southern refugia during the Pliocene and Pleistocene, and various morphologically cryptic lineage pairs are now in secondary contact at different locations throughout the species' range (Figure 1). Based on average mtDNA P_{net}-distances, Austin *et al.* (2004) estimated the mean divergence times among lineages to date to the Pliocene (~2 to 5 million years before present), more than sufficient time for sister species pairs to have evolved reproductive isolation (Lemmon, 2009), implying that we might expect varying degrees of reproductive isolation among lineage pairs. Spring peeper males produce single note advertisement calls that are between 91 and 280 ms in duration, and range between 2700 and 3200 Hz (Doherty and Gerhardt, 1984). These calls are easy to characterize sonographically (Wilczynski *et al.,* 1984), and females show strong ability to discriminate between conspecific and heterospecific calls of acoustically similar species (Gerhardt, 1973). Spring peepers thus represent a promising system to test whether lineages within a single taxon truly

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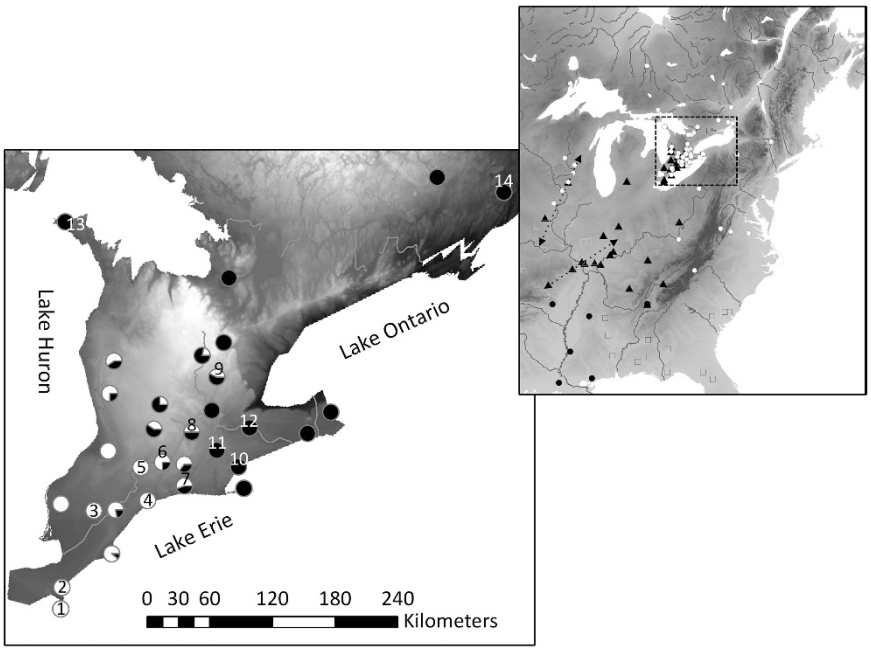

Figure 1 Map of Southwestern (SW) Ontario indicating proportional representation of Interior (white) and Eastern (black) mtDNA lineages. Locations for which nuclear microsatellite data are available are indicated with numbers (1–14) corresponding to information in Table 1 and Figure 2. Inset: Distribution of 6 mtDNA lineages (symbols) across North America (from Austin *et al.,* 2004) with the SW Ontario contact zone bordered by broken square. Also indicated by two-sided arrows are the two additional secondary contact zones (see Discussion).

represent emerging fully independent species, and whether time since divergence correlates with degree of reproductive isolation.

We focus on a previously described (Austin *et al.,* 2002) zone of secondary contact between Interior and Eastern mtDNA lineages of the spring peeper in southwestern Ontario, Canada (Figure 1). Secondary contact between these lineages occurred 10 000 to 15 000 years ago, following the most recent glacial retreat in this region (Austin *et al.,* 2002). Our objectives are to: (1) test for divergence in morphology and male advertisement call between lineages; (2) test whether females prefer calls of their natal lineages; (3) quantify the frequency of hybridization between lineages using nuclear DNA (nDNA) markers, including concordance or discordance in the shape of clines for different marker classes; and (4) test for character displacement of male traits, particularly calls, within population of mixed mtDNA lineages, hereafter referred to as the 'contact zone'.

MATERIALS AND METHODS

Study species

Spring peepers are small (20 to 30 mm snout–vent length) chorus frogs with females slightly larger than males. Mate selection by females is mediated primarily by male vocalizations (Forester and Czarnowsky, 1985). In North America, spring peepers are among the first frogs to breed in spring, and their breeding season is prolonged. In southern Ontario, Canada, male spring peepers typically call from early April until early June, with peak breeding in May.

Sampling design

We examined geographic variation in female preference, neutral DNA markers and acoustic and morphological traits of spring peepers (*P. crucifer*) in a zone of secondary contact in southwestern Ontario (Figure 1), consisting of admixture of Interior and Eastern mtDNA lineages. In total, 32 populations were sampled from 2003 to 2011 to obtain genetic, morphological, acoustic and female preference data (Figure 1 and Table 1).

We sampled 579 reproductive adults between April and June (2100 to 0200 h local time) collected across a 580-km straight-line sampling distance. Of these, 185 males from a total of 25 populations were used for call analysis: 10 mtDNA contact zone populations (breeding aggregations consisting of both Eastern and Interior lineages), 6 pure Interior populations (that is, no Eastern mtDNA) and 9 pure Eastern populations (that is, no Interior mtDNA). Full genetic and morphological analysis used data from 429 individuals (including 86 females) sampled from 4 previously delineated mtDNA contact zone populations, 5 pure Interior and 5 pure Eastern populations (Table 1). Of the 429 individuals, we were unable to amplify 6 spring peeper (4 male, 2 female) mtDNA sequences sampled within the contact zone and thus were left with 423 individuals.

DNA extraction, cytochrome *b* sequencing and microsatellite genotyping

DNA extractions were done using the DNeasy (QIAGEN, Mississauga, ON, Canada) Extraction Kit following the manufacturer's protocols. DNA concentrations were quantified using a Nanodrop ND 1000 spectrophotometer (Wilmington, DE, USA). Individuals were sequenced for a 692 base pair segment of cytochrome *b* (cyt *b*) using primers MVZ 15l and MVZ 18H (Moritz *et al.*, 1992) according to methods outlined in Austin *et al.* (2004). Briefly, 25 μl PCR cocktails contained 10× Fermentas reaction buffer (KCl), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.1 μM forward and reverse primer, 0.5 U of Taq Polymerase (Fermentas ThermoScientific, Burlington, ON, Canada) and 2 μl of DNA (5 to 20 ng μl^{-1}). Amplifications were performed in a GeneAmp PCR System 2700 (Applied Biosystems, ThermoScientific) using the following cycling profile: denaturation step of 3 min at 94 °C, followed by 45 cycles of 45 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C with a final extension of 5 min at 72 °C.

Cyt *b* PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen Inc., Chatsworth, CA, USA) before sequencing on an Applied Biosystems 3730 Analyzer (ThermoScientific, Robarts Sequencing Facility, London, ON, Canada). Sequences were aligned in BioEdit version 7.1.3 (Hall, 1999). We used a subset of 360 base pairs with 22 diagnostic single-nucleotide polymorphisms to identify mtDNA lineage affiliation for each individual.

We genotyped individuals at 11 microsatellite loci including Pcru05, Pcru10, Pcru11 and Pcru 32 (Degner *et al.,* 2009), and 7 additional markers developed for this study (Table 2); locus pairs Pcru11 and Pcru21, Pcru08 and Pcru24,

Table 1 Sampling information of populations from 2003 to 2011 with total number of individuals sampled located in brackets

Site name	*Collection years*	*Pop type*	*GPS coordinates*	*Data type*	*CFIT distance (km)*	*STRUCTURE Pop no.*
Pelee/Hillman[a]	2003, 2008–2010 (56)	Interior	41.971037–82.534279	G_{51}, M_{49}, C_{34}, F_7	580.76	1
Kopegaron[a]	2003, 2008–2010 (6)	Interior	42.07971–82.49243	G_3, M_3, C_6	567.58	2
Mosa Forest[a]	2004, 2010, 2011 (19)	Interior	42.656561–81.807461	G_{13}, M_{13}, C_6	483.45	3
Fingal[a]	2003, 2009–2011 (83)	Interior	42.671617–81.32635	G_{76}, M_{76}, C_7, F_{12}	453.98	4
PondMills	2011 (4)	Interior	42.945366–81.216774	G_4, M_4	427.04	5
ElginRd	2009 (4)	Contact	42.961887–81.014814	G_4, M_4,	413.66	6
Calton[a]	2003,2009–2011 (122)	Contact	42.7251–80.883783	G_{110}, M_{110}, C_{12}, F_{26}	420.68	7
Dereham[a]	2003, 2011 (20)	Contact	42.90885–80.834133	G_{17}, M_{17}, C_3	405.90	8
Starkey Hill[a]	2003, 2010, 2011 (45)	Contact	43.545624–80.156121	G_{38}, M_{38}, C_7, F_{11}	382.44	9
LPWREC	2011 (20)	Eastern	42.718358–80.351729	G_{20}, M_{20},	373.28	10
Vanessa[a]	2003, 2009–2011 (22)	Eastern	42.960443–80.397949	G_{15}, M_{15}, C_7, F_5	337.74	11
LaFortune	2009–2011 (41)	Eastern	43.092883–80.001717	G_{41}, M_{41}, F_{17}	318.76	12
BPNP	2009, 2010 (29)	Eastern	45.209134–81.56559	G_{29}, M_{29}, F_8	211.76	13
QUBS	2009 (5)	Eastern	44.496804–76.414847	G_5, M_5	0	14
Wainfleet[a]	2004 (9)	Eastern	42.922399–79.374779	G_9, C_9	NA	NA
Minesing[a]	2004 (5)	Eastern	44.410927–79.819107	G_5, C_5	NA	NA
Sudden Tract[a]	2003 (8)	Eastern	43.309191–80.38044	G_8, C_8	NA	NA
Chippawa[a]	2003 (7)	Eastern	43.053367–79.05427	G_7, C_7	NA	NA
Alton[a]	2003 (5)	Eastern	43.870733–80.0588	G_5, C_5	NA	NA
Port Dover[a]	2003 (10)	Eastern	42.783307–80.230408	G_{10}, C_{10}	NA	NA
Parrot Bay[a]	2003 (9)	Eastern	42.783307–80.230408	G_9, C_9	NA	NA
Harlowe[a]	2003 (10)	Eastern	44.787649–77.121965	G_{10}, C_{10}	NA	NA
N Guelph[a]	2003 (2)	Contact	43.586622, -80.231512	G_2, C_2	NA	NA
Vanisttart[a]	2004 (3)	Contact	43.16775–80.664817	G_3, C_3	NA	NA
Elice Swamp[a]	2004 (2)	Contact	43.4686–80.954883	G_2, C_2	NA	NA
Stapleton Tract[a]	2004 (6)	Contact	43.926917–81.355883	G_6, C_6	NA	NA
Hallet[a]	2003 (2)	Contact	43.65615–81.485533	G_2, C_2	NA	NA
Wildwood[a]	2004 (3)	Contact	43.267206–81.076355	G_3, C_3	NA	NA
Big Bend[a]	2004 (6)	Contact	42.64691–81.70833	G_6, C_6	NA	NA
Parkhill[a]	2004 (7)	Interior	43.17065–81.645283	G_7, C_7	NA	NA
Moore[a]	2004 (9)	Interior	42.801447–82.310658	G_9, C_9	NA	NA

Abbreviations: NA, not applicable; Pop, population.
The type of data collected for individuals within a sampling locale with subscript sample size is represented by G (genetic), M (morphology), C (male call) and F (female preference). GPS coordinates are in decimal degrees (latitude, longitude).
[a]Original 25 locales used for call/morphological analysis.

Table 2 Primer ID and GenBank Accession numbers, author affiliation, allele ranges and PCR reaction forward and reverse primer quantities for 11 microsatellite markers used during the genotyping of *P. crucifer* from 2008 to 2011

Primer ID	*Forward sequence (5′ to 3′)*	*Reverse sequence (3′ to 5′)*	T_a	*GenBank accession no.*	*Reference*	*Allele range*	*Repeat motif*	*Quantity PCR reaction primer*
Pcru32	CCCTACATAGGATTG	GTACACCCACCAAAAGTGCT	54	EF190904	Van Buskirk *et al.* (2006, unpublished)	121–163	$(CA)_{11}$	0.2 μl
Pcru10	GGGGGATGCAGAATT	CGGTTCCTATTGAAGAACA	54	EF190896	Degner *et al.* (2009)	187–213	$(CA)_{13}$	0.5 μl
Pcru05	CATTTATAAGCAGTGCAGAGAGG	TGCATTGATGTTTCTCATGG	54	EF190892	Van Buskirk *et al.* (2006, unpublished)	320–366	$(CA)_{13}$	0.5 μl
Pcru11	GGATATGCTCACATG	CCCAGATTCCAAGTGTTTTC	55	EF190897	Van Buskirk *et al.* (2006, unpublished)	112–168	$(GT)_{16}$	0.3 μl
Pcru21	TGGAGACATCATTGC	CCCTTGGTCCTGAATAGGTT	55	EF190900	Van Buskirk *et al.* (2006, unpublished)	210–264	$(CA)_{14}$	0.45 μl
Pcru09	GGGGGATGCAGAATT	CGGTTCCTATTGAAGAACA	54	EF190896	Degner *et al.* (2009)	125–159	$(CA)_{15}$	0.2 μl
Pcru14	GATCAGACAGTCTACAGTAATGAGGAG	CATAACACAGGGCAACCAAG	54	EF190899	Degner *et al.* (2009)	184–232	$(GT)_{13}$	0.3 μl
Pcru06	CATTTACAAACGGCACTGCTC	CCCCAGTCATCAGGAATACA	54	EF190893	Van Buskirk *et al.* (2006, unpublished)	186–236	$(GT)_{16}$	0.3 μl
Pcru12	TCAAATTGACCATCCATCC	GCCAGCCCCTATAGGATTAG	54	EF 190898	Van Buskirk *et al.* (2006, unpublished)	114–166	$(GT)_{13}$	0.5 μl
Pcru24	TGCCATGGGGATGTTATATG	CGAGCTATAGGAAAAGGCAGAG	54	EF190902	Degner *et al.* (2009)	96–146	$(CA)_{17}$	0.35 μl
Pcru08	CTAACCGTAGCAGGGAGGTTG	TAGGACTGAGGGAGGAGAGG	54	EF 190894	Van Buskirk *et al.* (2006, unpublished)	222–270	$(CA)_{12}$	0.5 μl

Pcru06 and Pcru12 and Pcru09 and Pcru14 were amplified together in duplexes, whereas loci Pcru05, Pcru10 and Pcru32 were amplified in a triplex reaction. Each 11 μl PCR reaction contained: 0.2 to 0.5 μl of forward and reverse primers (Table 2), 10× Fermentas reaction buffer (KCl), 1.5 mM $MgCl_2$, 10 mM dNTPs, 0.5 U of Taq Polymerase (Fermentas ThermoScientific) and 2 μl of DNA (5 to 20 ng μl^{-1}). Thermocycling conditions for Pcru09, Pcru14, Pcru06, Pcru12, Pcru24 and Pcru08 are outlined in Degner *et al.* (2009). Thermocycling conditions for Pcru32, Pcru10, Pcru05, Pcru11 and

Pcru21 following an initial denaturing (94 °C for 3 min) were as follows: 35 cycles of 94 °C for 15 s, 48 °C for 30 s and 72 °C for 40 s, followed by an extension of 15 min at 72 °C. PCR products were run on (2%) agarose gels (Invitrogen, Carlsbad, CA, USA) stained with 0.5 μg ml^{-1} ethidium bromide and visualized under ultraviolet light. Amplicons were genotyped using a Beckman Coulter (Mississauga, ON, Canada) CEQ 8000 capillary automated sequencer and scored using the CEQ 8000 Genetic Analysis System.

Clinal variation and quantifying hybridization

To test for introgression, we performed a cline analysis by first collapsing all locales to a single dimension using a straight-line distance in km between eastern-most (Eastern lineage) and western-most (Interior lineage) sampling points. Using the program CFIT Version 0.6 (Gay *et al.,* 2008), we estimated the best-fit shape parameters for the allele and haplotypic frequencies (f(x)) as a function of geographic distance (x). Clines were fitted for our 11 microsatellite markers and cyt *b* haplotypes, wherein each locus was reduced to a simple two-allele system using lineage-specific (either Eastern or Interior) compound alleles (Gay *et al.,* 2008) based on the first axis coordinates of a Multiple Correspondence Analysis implemented in GENETIX (Version 4.05; http://kimura.univ-montp2.fr/genetix/). We identified some private alleles across loci and found most alleles to be distinct between lineages. In instances where alleles are shared or incorrectly assigned, cline shape is typically flattened, and thus tests for the presence of clines are conservative (Gay *et al.,* 2008). We fitted the centre and width parameters of a logit cline for each microsatellite marker and the mtDNA, and then used Akaike information criteria to compare patterns of gene flow. We assessed four models: (1) unconstrained clines (difference in cline centres and widths), (2) cline centres coincident (geographic centres constrained), (3) cline widths concordant (widths constrained) and (4) cline centres coincident and widths concordant. All tests used a maximum centre value of 580 km (maximum transect distance) and used 1000 repetitions for 100 000 iterations (Gay *et al.,* 2008). Convergence was assessed by comparing the results of all models (replicated 10 times) with different random seeds. We used GENETIX to test for linkage disequilibrium between pairs of loci for each population ($P<0.05$). Low levels of linkage disequilibrium are expected with free recombination between parental genomes, whereas high linkage disequilibrium levels are expected under instances of rare hybridization events, hybrids with low fitness and/or low recombination (Harrison, 1993).

We used STRUCTURE (version 2.3.3; Pritchard *et al.,* 2000) to distinguish between mixed versus pure lineage individuals, using a Markov Chain Monte Carlo algorithm to infer population structure from multilocus genotype data. Individuals were assigned to clusters that best met the assumptions of Hardy–Weinberg equilibrium and linkage equilibrium, identifying hybrids based on allele frequencies. We assumed admixture, correlated alleles, no prior population information and used 10 iterations of 5×10^5 steps of the Markov Chain (preceded by a burn-in period of 25×10^4 steps). We ran STRUCTURE testing K (number of genetic clusters) from 1 to 14, with 10 replicates for each K. STRUCTURE outputs were entered into Structure Harvester (Earl and vonHoldt, 2011), implementing the Evanno *et al.* (2005) method for identifying the most probable value of K.

To assess the power of 11 microsatellites to differentiate between 'pure' and 'admixed' individuals, we simulated four classes (F1, F2 and backcross individuals) each consisting of 100 hybrid individuals. Simulations were conducted using HYBRIDLAB version 1.0 (Nielson *et al.,* 2006) utilizing 30 'pure' lineage individuals from each of the Interior and Eastern lineages ($0.95\geq q\leq0.05$) sampled from multiple populations that were located at least 100 km from the contact zone (populations with both haplotypes). These simulated genotypes (50 randomly chosen per category) were subsequently used for a STRUCTURE analysis ($K=2$ following the methods outlined previously), and the proportion of correctly identified pure and hybrid individuals were quantified.

Using cyt *b* sequence data, we also refined formerly calculated (Austin *et al.,* 2002; 2004) lineage divergence times that were based on mtDNA sequence *P*-distances by estimating TMRCA (time to most recent common ancestor) for all major spring peeper clades using the programs BEAUti and BEAST v1.4.8 package (Drummond and Rambaut, 2007; Supplementary Appendix S1).

Testing for character divergence and displacement

We measured snout–vent length, head width, tibia length, foot length, femur length and radioulna length (±0.2 mm) using digital callipers, and estimated mass using a 10 g spring Pesola scale on live individuals (Baar, Switzerland). Measurements were taken on the right side of the frog and all were taken by KAS. We used multiple approaches to test for morphological differences between pure and mixed populations, and among Eastern, Interior and hybrid genotypic classes in the Ontario transect. First, for each variable and for each sex separately, we tested for differences among groups using one-way analysis of variance (Supplementary Table S1), correcting for false discovery rate (Benjamini and Hochberg, 1995) and assuming a critical value of false discovery of 0.25. Second, we used multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA) to diagnose differences among groups (Supplementary Table S2). Because body size is important in anuran sound reception and production (Gerhardt, 1994), we also assessed morphological differences for each sex separately using a multivariate component of body size derived from principal components analysis (PCA; see Supplementary Tables S1 and S3).

Parental calls were characterized for localities comprising pure lineage individuals based on cyt *b* data and our understanding of where lineages come into secondary contact based on mtDNA lineage overlap. To reduce sampling biases in timing of data collection, we systematically alternated dates for sampling mtDNA populations. All males that were calling during the spring peeper's short breeding period were assumed to be reproductively active. We located males at night and recorded their advertisement calls using a Marantz (Kleinberg, ON, Canada) PMD660 and a Sennheiser (Point Claire, PQ, Canada) ME67 directional microphone held ~1 m from the calling individual. We analysed at least 10 consecutively produced advertisement calls per calling male that were clear and distinct from other nearby calling individuals and used the mean to represent each individuals call. We recorded air and water temperature using a Raytek (Santa Cruz, CA, USA) Minitemp MT6 laser thermometer at each calling site, noting the position details of the focal male. After recording, males were captured by hand, measured, toe-clipped and immediately released. We measured nine call parameters previously identified as being important for species recognition and/or sexual selection in chorus frogs (Lemmon, 2009; see Supplementary Figure S1) using SYRINX (v2.6; www.syrinxpc.com) and both oscillograms and spectrograms (Blackman, 2.04 ms, FFT window length = 1024). Eight of the nine parameters (maximum frequency, minimum frequency, time to maximum frequency (rise time), time to minimum frequency (fall time), carrier length, call duration, call rate and call interval, but not delta frequency) varied significantly with temperature (all $P<0.05$; Supplementary Table S4); thus, we used residuals from linear regressions of these parameters on temperature for subsequent DFA (Supplementary Table S5) and MANOVA. Many call parameters also vary with body size in frogs (McClelland *et al.,* 1996) and body size itself may be a target of selection; thus, we subsequently ran a DFA on variables corrected for snout–vent length to test whether acoustic parameters differed despite possible morphological distinction between lineages (Supplementary Table S5). DFA may distort multivariate space and exaggerate differences among *a priori* defined groups (Mitteroecker and Bookstein, 2011); thus, we also performed a PCA on these same call data and subsequently tested for differences in PC scores among groups using one-way ANOVAs and Tukey–Kramer *post hoc* tests where appropriate.

Prezygotic preference experiments

We performed phonotaxis experiments using antiphonally presented natural calls in a standardized test arena to quantify female preference for male calls. Specifically, we tested for female preference of males from their own mtDNA lineage, or for males possessing a divergent mtDNA haplotype. We used females from the contact zone and from the geographic extremes of our sampling area. For stimuli, we used natural calls sampled in pure populations from three males per lineage with average lineage snout–vent length, whose calls were recorded at 16 °C (temperature at which females were tested; Lemmon, 2009). These calls were combined into multiple digital files with all possible contrasting call pairs to reduce biases. Stimuli were played using an audio editor program (Audacity Version 2.2.5; http://audacity.sourceforge.net/), and broadcast through two

opposing speakers (Saul Mineroff Field Speakers SME-AFS, Elmont, NY, USA). Stimuli were presented antiphonally at 85 dB sound pressure level as measured at the centre of the testing arena (see below), mimicking natural amplitude attenuation. Call stimuli for each lineage were normalized to the same call interval (1 call s^{-1}) and amplitude (±2 dB) during all experiments. We randomized which lineage-specific call was presented first and continuously played a background chorus at 10 dB below that of the stimuli.

Amplexed and/or gravid females for phonotaxis experiments were caught in pure and mixed populations and transported to a field station within 2 h of collection site (Point Pelee National Park, Bruce Peninsula National Park, Long Point Waterfowl Research and Education Centre, or Queen's University Biological Station). After >2 h of acclimation in the dark, females were individually placed into the testing arena, a white 142 cm diameter wading pool filled with ~6 cm of aged tap water and perching sticks (Lemmon, 2009). Within a dark room, female spring peepers were placed at just over 50 cm away from both speakers within a plastic container with stimuli played for 2 min before release. Container lids were removed remotely and female movements were recorded using a Sony Handycam DCR HC21 Mini DV HD camcorder with a Sony (New York, NY, USA) HVL IRM Infrared light. Approach to within 10 cm radius of a speaker emitting stimuli was considered a positive response (Lemmon, 2009); the response time was also recorded. Females that did not respond to either stimulus after 15 min or that swam around the perimeter of the pool instead of making a directional choice were considered unresponsive. We did not inject females with luteinizing hormone to induce receptivity, and each female was only tested once to reduce false positives and eliminate pseudoreplication. After trials were completed, females were toe clipped and genotyped to designate mtDNA lineage ancestry, because morphology alone does not distinguish between lineages or between pure and hybrids categories. All females were tested on the night of capture and released at their capture site the following evening.

RESULTS

Clines

Logit cline analysis (Figure 2a) on composite alleles (GENETIX MCA first axis eigenvalue = 1.98) indicated that the third model (concordant cline widths but non-coincident centres for nDNA and mtDNA; widths constrained) best fits our data (Supplementary Table S6). The second best model (model 1) included the unconstrained clines (cline centres and widths were dissimilar between genetic markers). The worst fitting model was model 4, spatially coincident clines with concordant widths for both genomes. The estimated cline width for both genomes was 90.9 km, with centre values for the microsatellites ranging from 335 to 580 km from the eastern-most point on transect. Our cyt *b* marker cline centre was estimated to be at 395 km (Figure 2a). Linkage disequilibrium was highest near the mtDNA contact zone and lowest in peripheral transect populations (Supplementary Table S7).

Our STRUCTURE analysis supported the presence of two lineages (Supplementary Table S8) in secondary contact ($K = 2$). HYBRIDLAB simulations based on putative 'pure' lineage genotypes produced q-values (hereafter 'q'; 0 = pure Eastern, 1 = pure Interior) ranging from 0.86 to 0.96 for Interior, 0.043 to 0.11 for Eastern, 0.31 to 0.68 for F1 hybrids, 0.21 to 0.92 for F2 hybrids, 0.49 to 0.94 for Backcross Interior (F1 hybrid × Interior) and finally 0.06 to 0.44 for Backcross Eastern (F1 hybrid × Eastern). Overlap in q from our simulated data precludes us from distinguishing among F1, F2 or backcross individuals in observed data; henceforth, we designate pure Interior individuals with a $q > 0.85$, pure Eastern with a $q < 0.15$ and 'hybrids' as $0.15 < q > 0.85$).

Hybridization and introgression were asymmetrical across the mtDNA contact zone. Four sites that contained both Interior and Eastern haplotypes had high proportions of pure Eastern individuals and entirely lacked pure Interior individuals. Using our defined hybrid class ($0.15 > q < 0.85$), results from STRUCTURE suggest that Eastern lineage individuals ($n = 79$) within the contact zone were either of pure Eastern ancestry (Eastern nDNA genotypes; 36.4% males and 29.2% females) or hybrids (63.6% males and 70.8% females; Figure 2b and Supplementary Table S9). Despite finding 80 individuals with Interior mtDNA within the contact zone, all were diagnosed as being of hybrid origin (Figure 2band Supplementary Table S9).

The TMRCA mean estimate for coalescence of the entire species using 1% DNA sequence divergence was 11 million years ago (95% highest probability densities 6.83 million to 15.4 million). Based on

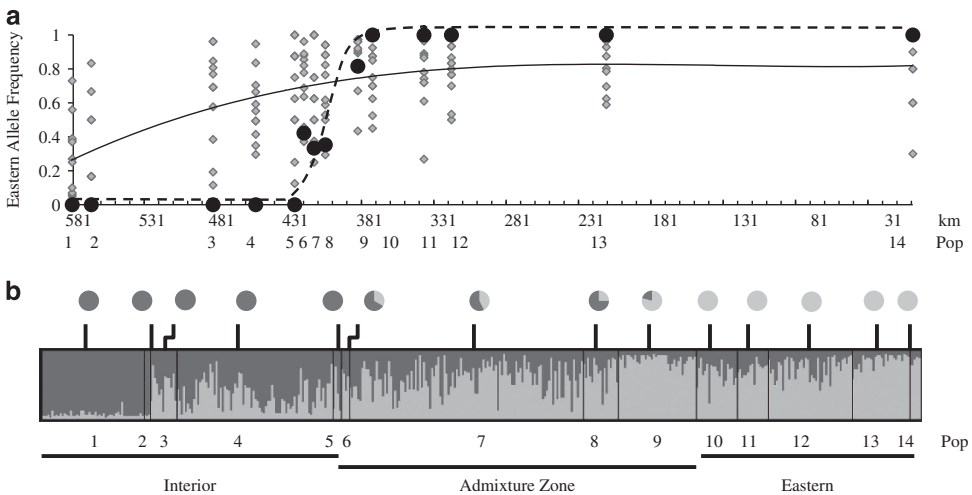

Figure 2 (**a**) Frequency of 11 Eastern spring peeper compound microsatellite alleles (grey diamonds) and Eastern cyt *b* mtDNA haplotypes (black circles) across the contact zone from 0 km (Queen's University Biological Station near Kingston, Ontario) to 580 km (Point Pelee National Park/Hillman Marsh); polynomial trend lines (solid = nDNA, dashed = mtDNA) over geographic distance. Population (Pop) numbers below x axis correspond to STRUCTURE analysis for geographic locale comparison. (**b**) Bar plot of admixture coefficients resulting from STRUCTURE analysis with $K = 2$. Individuals are represented as vertical bars partitioned into two segments the length of which is proportional to the estimated membership in the two clusters (dark grey = Interior, and light grey = Eastern). Pie charts above show proportion of Interior or Eastern cyt *b* haplotypes for each sampled locale. Admixture of lineages is present in sites 6 through 9; see Table 1.

TMRCA analysis, the Interior and Eastern lineages implicated in the southwestern Ontario mtDNA contact zone began diverging in the Pliocene ~4.94 million years ago (95% highest probability densities HPD 3.06 million to 6.96 million) (Supplementary Appendix S1).

Morphological and call character displacement?

Independent of sex, the MANOVA revealed significant morphological differences among different genetic groups (Wilks' $\lambda = 0.843$, $F_{4, 369} = 1.913$, $P < 0.001$; Figure 3a). DFA showed that frogs from pure Eastern populations were generally shorter but heavier than frogs from pure Interior populations. Hybrid individuals also differed from pure Interior and pure Eastern genotypes. Analysis of body size (PC1 scores; Supplementary Table S3) revealed that hybrid females were smaller than pure lineage females within the mtDNA contact zone (that is, pure Eastern females) and females from either lineage at non-contact zone locations ($F_{4, 75} = 3.95$, $P = 0.006$; Supplementary Figure S2A). This smaller size was pronounced in hybrid females with Eastern haplotypes. Body size (PC1) in males, however, did not differ significantly across populations or lineages ($F_{4, 318} = 0.831$, $P = 0.528$; Supplementary Figure S2B), suggesting overall body size differences between lineages are primarily driven by females. Males, however, did exhibit significant differences in snout–vent length (among other traits), with hybrids of the Eastern mtDNA lineage and pure Eastern individuals sampled showing the smallest size ($F_{4, 429} = 5.13$, $P < 0.001$; see Supplementary Table S1). Once more, male hybrids were significantly shorter (snout–vent length), whereas females had significantly smaller mass (Supplementary Table S1).

A MANOVA also revealed significant differences among male call parameters (Wilks' $\lambda = 0.606$, $F_{4, 185} = 2.70$, $P < 0.001$; Figure 3b). Males from our two lineages sampled outside of the mtDNA contact zone had significantly different advertisement calls, with Eastern males having calls with a significantly shorter call rise ($F_{4, 185} = 12.72$, $P < 0.001$), shorter fall time ($F_{4, 185} = 12.73$, $P < 0.001$), higher carrier length ($F_{4, 185} = 8.12$, $P < 0.001$) and a borderline, nonsignificant higher top frequency ($F_{4, 185} = 2.25$, $P = 0.066$) when compared with Interior males. Call attributes for mtDNA contact zone pure Eastern males also differed significantly from their pure population counterparts showing no overlap in 95% confidence ellipses in the DFA (Figure 3b); their calls had significantly shorter call intervals ($F_{4, 185} = 2.43$, $P = 0.05$) than pure Eastern, pure Interior or hybrid males. Again, we found no pure Interior individuals in the contact zone, but male hybrids with Interior haplotypes had calls that were intermediate compared with the calls of the two pure parental lineages (Figure 3b); Eastern hybrid males had calls similar to those produced by Eastern males from outside the mtDNA contact zone. Importantly, MANOVA differences in vocalizations were apparent even after controlling for body size (snout–vent length; Wilks' $\lambda = 0.611$, $F_{4, 185} = 3.20$, $P < 0.001$; Supplementary Table S5). PCA analysis similarly demonstrated significant call differences among population types; Interior males from pure populations showed higher, and Eastern males from both pure and mixed populations showed significantly lower, PC1 scores ($F_{4, 193} = 5.90$, $P < 0.001$). After controlling for body size, PCA analysis on male calls similarly showed significantly higher PC1 scores for Interior males from pure populations compared with all other genetic groups ($F_{4, 185} = 9.70$, $P < 0.001$).

Female preference

Of the 86 females collected between 2008 and 2011, 7 were not gravid and/or reproductively active and were thus not tested for call preference (however were still included in morphological and genetic analyses). Of the remaining 79 females, we were unable to amplify the mtDNA sequences for 2 individuals found within the mtDNA contact zone, thus further reducing female sample size to 77.

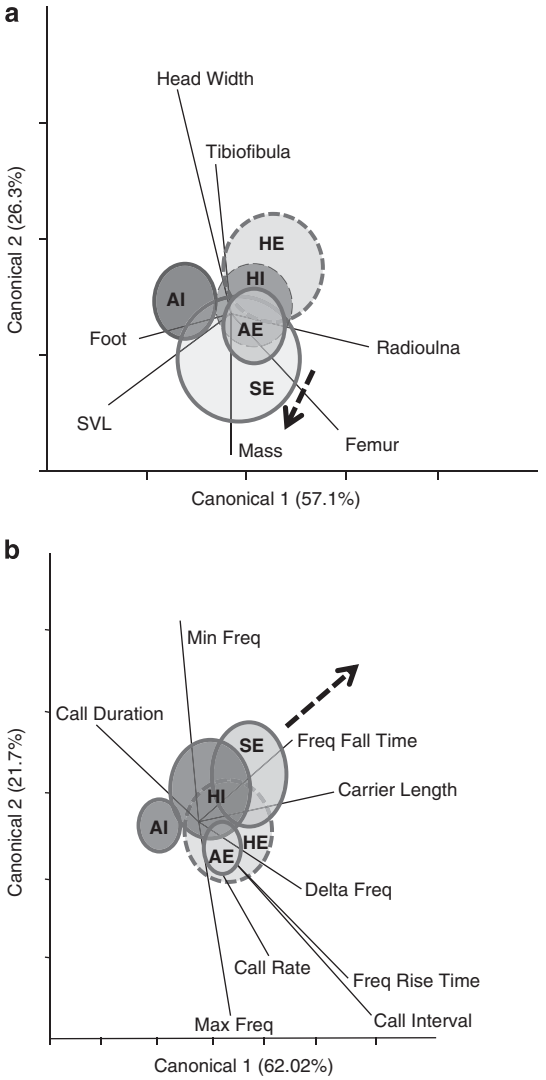

Figure 3 Biplot of the first two axes from a DFAs with 95% confidence ellipses of pure Eastern and Interior individuals in allopatry (pure populations; AE and AI) and sympatry (mixed populations; SE; no SI individuals were identified), and hybrids of different haplotypes (HE and HI) for (**a**) external morphology and (**b**) male advertisement calls. Proportion of total variation explained is represented on each axes; arrow highlights the direction of displacement for pure Eastern males in sympatry (SE). Males with Interior haplotypes are shown in dark grey circles and males with Eastern haplotypes are show in light grey circles (solid lines for genetically pure and dashed lines for hybrid individuals).

Of the 77 tested females for male advertisement call preferences, 13 were from pure Interior, 19 from pure Eastern and 45 females from within mtDNA admixed populations (because of cryptic phenotypic differences between lineages and hybrids, we classified individuals *post hoc* using mtDNA and nDNA data). Of the 77 females, 28 (36.4%) provided a scorable response. Responses were highest in pure Interior populations (6/13; 46.2%), intermediate in mixed populations within the mtDNA contact zone (17/45; 37.8%) and lowest in pure Eastern populations (5/19; 26.3%). Within mixed populations we found little difference in response rate between pure Eastern (4/17; 24% and

Eastern hybrid (5/17; 29.4%) females compared with Interior hybrid females (8/17; 47%). Pure Eastern females within the contact zone showed a shorter mean response time to stimuli (85.8 s) compared with Eastern hybrid (141.2 s) and Interior hybrid females (171.4 s) that showed a greater response lag but not significantly so ($F_{2,16}=1.54$, $P=0.26$). Interior females from pure populations exhibited a preference for Interior stimuli (83.3%). Pure Eastern population females also demonstrated a slight preference for Interior stimuli (60%). Contact zone pure Eastern females, on the other hand, consistently (100%) chose vocalizations from Eastern males over Interior ones (Pearson's $\chi^2=8.15$, d.f.$=3$, $P=0.04$). Moreover, pure Eastern females within the contact zone demonstrated a significantly higher preference than their Eastern pure population counterparts (two-tailed Fisher's exact test, $P=0.047$). Female hybrids showed near equal preference for stimuli (53% preferred Eastern); however, when separated into their respective mtDNA lineage groupings, female hybrids with Eastern haplotypes favoured Eastern stimuli (80%) and female hybrids with Interior haplotypes exhibited equal preference for either stimulus (50%).

DISCUSSION

We found discordant clines in nDNA and mtDNA markers, differences in morphology and male vocalizations between mtDNA lineages and significant differences in female preference for lineage-specific male calls among pure population and contact zone females. The asymmetrical introgression of mtDNA indicated by non-coincident clines in mtDNA and nDNA within the previously defined mtDNA contact zone is most likely caused by unidirectional hybridization or selection against hybrids. Narrower mtDNA versus nDNA clines, as we have identified here, suggest various possibilities including: (1) nuclear introgression and/or differences in hybrid viability, (2) fertility differences between the sexes (Toews and Brelsford, 2012), (3) differences associated with effective population size of mtDNA versus nDNA or (4) pronounced differences in sex-biased dispersal.

In taxa where females are heterogametic, mtDNA clines are typically narrower with expected geographical discordance or non-coincidence between nDNA and mtDNA markers (Toews and Brelsford, 2012). Although heterogamy has not been reported for *Pseudacris*, and thus Haldane's Rule (Haldane, 1922) cannot be invoked, a higher fitness cost to Eastern hybrid females would be consistent with the asymmetrical introgression seen here. Indeed, female but not male hybrids are smaller than their pure lineage counterparts and we know that fecundity correlates positively with body size in female anurans both within (Camargo *et al.,* 2005) and among species (Prado and Haddad, 2005). Frogs exhibit indeterminate growth (Zug *et al.,* 2001), and smaller body size may also correlate with lower life expectancy for female hybrids.

Hybrid females collectively did not show any preference for the different calls of Interior or Eastern males. Separately however, Eastern hybrid females preferred calls of males from their own lineage, whereas Interior hybrid females exhibited no mean preference, further implying that asymmetrical mating may shape the contact zone. Asymmetrical female preference alone can cause hybrid zone movement (Shapiro, 2001). For example, if one parental population produces more pure offspring than another when in secondary contact, in subsequent generations there will be a replacement of the latter's genome, regardless of the fitness of hybrids *per se* (Bella *et al.,* 1992; Buggs, 2007). We found some evidence for increased female preferences for their parental lineage call within the contact zone consistent with selection against hybridization.

Our morphometric, call and behavioural data are also consistent with a hypothesis of selection against hybridization, at least in females of the Eastern lineage. The significant divergence in male call and morphology between pure populations is stronger within the mtDNA contact zone, suggesting that this exaggerated displacement response is not merely a subset of already existing character states in peripheral populations (Noor, 1999; Coyne and Orr, 2004). Furthermore, female preference in frogs from pure Eastern populations appeared to be lower than those sampled in the contact zone, consistent with RCD. Because our two lineages do hybridize, it is likely that such divergence would be driven by selection to avoid hybridization itself and not by signal interference avoidance (Noor, 1999). Indeed, congeners of *P. crucifer* also show RCD in male call traits and female preference in sympatry as a means of avoiding maladaptive hybridization (Lemmon, 2009).

Discordance between mtDNA and nDNA in hybrid zones, where the former is narrower than the latter, can reflect the increased rate of genetic drift expected in mtDNA relative to nDNA (Moore, 1995). The differences in the clinal patterns of mtDNA and nDNA may also simply be caused by sex differences in dispersal alone (Rheindt and Edwards, 2011), where male biased dispersal would allow for the persistence of a narrow band of divergent mtDNA lineages. A study of spring peepers in Michigan (Delzell, 1958) close to our focal contact zone showed that post-breeding home range sizes and dispersal distances were approximately equal for both sexes. Pronounced differences in gene flow resulting from sex-biased dispersal were also not evident in our data (see Supplementary Appendix S2 for *post hoc* analysis of sex-biased dispersal).

In the absence of quantified fitness consequences in hybrid *P. crucifer* (Stewart and Lougheed, 2013), plausible alternatives for the patterns that we observed require discussion. For example, asymmetrical mtDNA introgression may be caused by local adaptation, mtDNA–nDNA coevolution or selective sweeps of universally favoured mutations (Irwin *et al.,* 2009). The lack of obvious environmental gradients geographically coincident with this zone of secondary contact (Williams, 2009) and our use of putatively neutral loci does not support the local adaptation hypothesis. More importantly, asymmetrical mtDNA introgression alone via the above mechanisms cannot explain our observed geographical patterns in morphology, male call or female preference. Even if we posit pleiotropic effects of mtDNA on morphology and acoustics in pure Eastern population individuals, these features should not be exaggerated in the contact zone.

Ecological effects to reduce niche overlap (Ecological Character Displacement) may concomitantly change mate recognition signals in sympatry (Jang *et al.,* 2009). Nevertheless, when correcting for body size between lineages we still see strong evidence for call divergence in pure peripheral populations, and apparent RCD within the contact zone in acoustic traits. Indeed, the call parameters that best distinguish between males in different populations are primarily call rise and fall times, attributes unrelated to body size in our species (Supplementary Table S5). Furthermore, morphometric differences are largely driven by differences in females, supporting the contention that call divergence and displacement are not simply the result of ecological constraints. In effect, this sex asymmetry in morphological traits may distinguish selection against hybridization from alternative mechanisms (Coyne and Orr, 2004).

Our data are thus consistent with the hypothesis that RCD has occurred between these two lineages now in secondary contact, with selection against hybridization causing the observed pattern of female preferences, non-coincident clines/asymmetrical introgression,

morphologically distinct hybrids and hybrid females showing asymmetrical preference. Hybrid males show gametic (Wang, 2012) and behavioural (alternative mating tactic) dysfunctions (Stewart, 2013), in addition to some observations of higher tadpole mortality in hybrids under stressful developmental periods (Stewart and Lougheed, 2013), although this latter finding requires confirmation.

Genetic patterns within two geographically independent *P. crucifer* contact zones (Supplementary Appendix S3) among lineages with deeper TMRCA (southeastern Missouri to southern Illinois and northeastern Missouri to Wisconsin; Supplementary Appendix S1) imply that secondary contact and subsequent introgression may be geographically pervasive and important in determining evolutionary outcomes. Examination of the northeastern Missouri to Wisconsin contact zone (Supplementary Figure S3-1A), for example, reveals patterns of introgression between the Interior and Eastern lineages that are similar to those of the focal Ontario contact zone. However, Western individuals within this same transect show little to no signature of introgression from either Interior or Eastern lineage populations despite geographic proximity and evidence of populations that show mtDNA admixture. Similarly, within the southern Illinois contact zone we see no marked introgression between Interior and Western populations (Supplementary Figure S3-1B), suggesting that time since divergence (8.92 mya) may indeed relate to factors that play a role in the ability for spring peeper lineages to hybridize.

CONCLUSION

A disproportionate number of studies on the mechanisms underlying the evolution of reproductive isolation have focused on either *a priori* diagnosed species or those with overt ecological disparities, whereas comparatively few studies have approached the same questions at the intraspecific level where differences among lineages may be subtle or cryptic. Secondary contact zones between well-defined phylogeographic lineages as we examined here provide unique opportunities to investigate processes during the earliest stages of speciation. Teasing apart the true nature of divergence is an important step in identifying the processes that contribute to the evolution of reproductive isolation, especially in those organisms where mate recognition traits are tightly linked to morphology. Our discovery of non-coincident clines in mtDNA and nDNA, call and morphological divergence between our two focal lineages, together with displacement in male advertisement calls, and female preferences within the hybrid zone are consistent with RCD, although more work is needed to verify such an assertion. Certainly, our results imply either unidirectional hybridization or some form of selection against hybrids. Our work highlights the benefit of a multi-character, integrative approach to studying divergence at the early stages of speciation. It also illustrates the utility of using phylogeographic perspectives in providing a genealogical and historical framework for studying processes of speciation, as well as the impact of dynamic fragmentation histories and secondary contact on evolutionary trajectories.

DATA ARCHIVING

Sequence data have been archived in GenBank (see Austin *et al.*, 2004). Genotypic, acoustic, morphological and preference data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.8h06d. DNA microsatellite data can also be found in the Queen's University Biological Station Archive: http://dataverse.scholarsportal.info/dvn/dv/QUBS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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