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HYBRIDIZATION LEADS TO SENSORY REPERTOIRE EXPANSION IN A GYNOGENETIC FISH, THE AMAZON MOLLY (*POECILIA FORMOSA*): A TEST OF THE HYBRID-SENSORY EXPANSION HYPOTHESIS

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Expansions in sensory systems usually require processes such as gene duplication and divergence, and thus evolve slowly. We evaluate a novel mechanism leading to rapid sensory repertoire expansion: hybrid-sensory expansion (HSE). HSE occurs when two species with differently tuned sensory systems form a hybrid, bringing together alleles from each of the parental species. In one generation, a sensory repertoire is created that is the sum of the variance between parental species. The Amazon molly presents a unique opportunity to test the HSE hypothesis in a "frozen" hybrid. We compared opsin sequences of the Amazon molly, *Poecilia formosa*, to those of the parental species. Both parental species are homozygous at the RH2–1 locus and each of the four long wavelength sensitive loci, while *P. formosa* possess two different alleles at these loci; one matching each parental allele. Gene expression analysis showed *P. formosa* use the expanded opsin repertoire that was the result of HSE. Additionally, behavioral tests revealed *P. formosa* respond to colored stimuli in a manner similar or intermediate to the parental species *P. mexicana* and *P. latipinna*. Together these results strongly support the HSE hypothesis. Hybrid-sensory repertoire expansion is likely important in other hybrid species and in other sensory systems.

KEY WORDS: **Evolutionary genomics, gene duplication, opsin, Poeciliidae, sensory system, visual system.**

The evolution of sensory systems is generally driven by selection acting upon variation arising through gradual intragenomic processes such as mutation, gene duplication, and recombination (Horth 2007). More rarely, novel variation in sensory systems could also arise through more rapid intergenomic processes such as hybridization, although empirical evidence for such a process has not yet been demonstrated.

We suggest that hybridization, a previously unexplored mechanism behind sensory repertoire expansion, could act to rapidly combine variation accumulated in two different genomes, often from different environments, into a single organism. By bringing together variation from two organisms in one mating event, hybridization expands the sensory repertoire much more quickly than more traditional models of duplication and divergence. We term this process the hybrid-sensory expansion (HSE) hypothesis (outlined in Box 1). A diploid F1 hybrid receives one allele of each sensory locus from both parental species (Dowling and Secor 1997; Lampert and Schartl 2008). The HSE hypothesis predicts that if the parental species' sensory repertoires are differentially tuned, then the sensory repertoire of the hybrid will be different than that of either parental species. When a hybrid species possesses a sensory repertoire, which is a combination of

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Box 1. Hybrid Sensory Expansion (HSE): hypotheses and predictions.

Hybrid-Sensory Expansion (HSE) Hypothesis

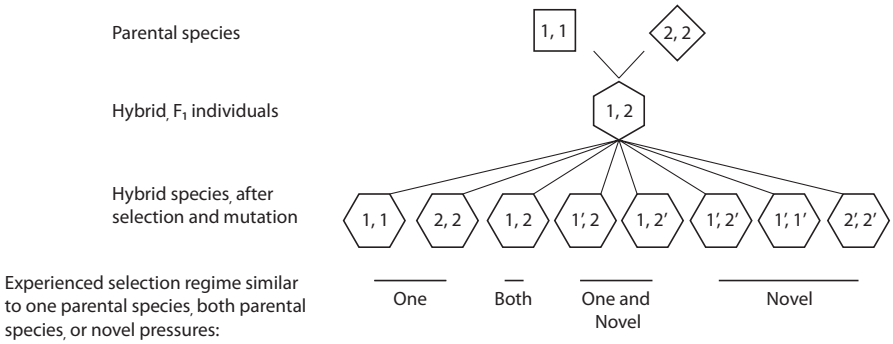
We propose that when a hybrid is formed between two parental species (P1 and P2) possessing functionally diverged sensory alleles (1 and 2) at the same autosomal locus, then the hybrid will be heterozygous, possessing the sensory alleles of both P1 and P2. Bringing together sensory alleles from P1 and P2 provides the hybrid with a combined sensory repertoire of both P1 and P2 (tested in this manuscript). While sensory expansion normally depends upon relatively slow evolutionary processes such as gene duplication and divergence, HSE occurs in a single generation. The expanded sensory repertoire may then be shaped by selection.

$$P1_{1,1} \times P2_{2,2}$$

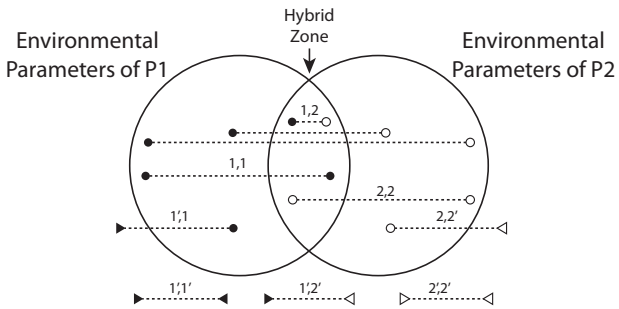
$$H1_{1,2}$$

HSE Predictions

The resulting sensory repertoire of the hybrid can be shaped by selection from various aspects of life history including: sexual selection, prey detection, predator detection, etc. The resultant sensory repertoire could resemble the sensory system of one, both, or neither parental species if the selective regime experienced by the hybrid resembles those experienced by either or both parental species or is entirely or partially novel.



Sensory systems are the tools by which organisms perceive and interact with their environment. Changes to the sensory system can change the environmental parameters under which an organism can survive. Sensory alleles inherited from parental species P1 and P2 have been tuned to facilitate interaction with the environmental parameters of P1 and P2 respectively. First generation hybrids have sensory alleles from both parental species suggesting they could live in the environments of P1 and P2. Depending upon the direction of selection the set of environmental parameters available to the hybrid species could decrease, or shift to a new set of parameters. Selection working to maintain the full set of P1 and P2 alleles in the hybrid species would be observed as heterozygote advantage and could explain behavioral differences within populations of hybrid species as some individuals would be homozygous for each of the parental sensory alleles. Similarly, different populations of hybrids could experience selection pressures in different directions; matching P1 or P2.



the variation present in the two parental species, selection may act on that variation. When a hybrid lives in a similar selective environment to the parental species, selection is likely to maintain the sensory repertoire to be more similar to either or both of the parental species, which could impact hybrid fitness.

The sensory repertoires of species capable of color vision are often tuned to take advantage of their specific spectral environments (Carleton and Kocher 2001; Endler et al. 2005; Fuller et al. 2005). Color vision is mediated via cone cells in the retina of the eye (Loew and Lythgoe 1978). The peak spectral sensitivity (λ_{max}) of a cone cell is determined by its visual pigment, consisting of a chromophore coupled with a transmembrane protein called an opsin, which is maximally sensitive to a specific wavelength of light (Yokoyama 2000, 2002). The fishes in the family Poeciliidae possess excellent color vision (Schwanzara 1967; Houde 1987; Anstis et al. 1998; Grether et al. 2005) and have undergone extensive gene duplication and subsequent differentiation in their long wavelength sensitive (LWS) opsin genes. Thus, some poeciliid species possess the largest known opsin repertoire among vertebrates (Hoffmann et al. 2007; Windsor and Owens 2009; Watson et al. 2011).

The Amazon molly (*Poecilia formosa*) is a member of the Poeciliidae family and is the result of a hybridization event between a female *P. mexicana* and male *P. latipinna* (Hubbs and Hubbs 1932; Avise et al. 1991; Schartl et al. 1995) that likely occurred once about 120,000 years ago (Stöck et al. 2010). *Poecilia formosa* reproduce through gynogenesis (Kallman 1962; Schlupp 2005), a form of clonal reproduction in which a diploid egg is created through mitosis. During this process, the development of the egg is triggered by the presence of sperm from a sexual species. However, the sperm's genetic material is almost never incorporated into the egg (Beukeboom and Vrijenhoek 1998; Schlupp and Riesch 2011). This unusual form of reproduction has led some people to call *P. formosa* "frozen F1s" (Vrijenhoek 1979), because they are predicted to still possess full haploid genomes from each parental species. Previous work on the cone cells of the parental species *P. mexicana* and *P. latipinna* show that they are tuned to different wavelengths of light in ranges characteristic of the RH2–1/LWS class of opsins, whereas the cone cells of *P. formosa* span the ranges of the parental species (Körner et al. 2006). Both of these classes of opsins function in the cone cells to provide photopic (color) vision. While RH2–1 typically detects greens and yellows, the LWS opsins detect greens, yellows, reds, and oranges (Yokoyama et al. 2008). The maintenance of the "F1 genotype" together with the potential of an expanded opsin repertoire makes specific predictions about the sensory repertoire of *P. formosa* allowing a formal evaluation of the HSE hypothesis.

We tested the HSE hypothesis by comparing RH2–1 and LWS opsin sequences of *P. formosa* to those of its parental species, *P. mexicana* and *P. latipinna*. The HSE hypothesis predicts that *P. formosa* RH2–1 and LWS opsin alleles should be more similar to one of the parental species (*P. mexicana* or *P. latipinna*) than they would be to each other, and that this combination of diverged parentally derived opsins would result in a unique, differentially tuned sensory system. We compared genomic sequence of RH2–1 and the four LWS opsins to evaluate our hypothesis that there is an expanded RH2–1/LWS opsin repertoire in *P. formosa* due to one chromosome coming from the maternal ancestor, *P. mexicana*, and the other chromosome coming from the paternal ancestor, *P. latipinna*. To verify that the hybrid species uses its expanded opsin repertoire, we identified which of the opsin alleles were being expressed in the eyes of *P. formosa*. In addition, we identified behavioral differences in response to color stimuli between *P. formosa* across these three species. We conclude by interpreting our findings of expanded opsin repertoire, expression profiles, and behavioral differences as support for the HSE hypothesis in light of experimental data on spectral tuning of cone cells in *P. formosa* and its parental species (Körner et al. 2006).

Materials and Methods

SAMPLE PREPARATION, PCR, CLONING, AND SEQUENCING

DNA was extracted from tissue samples of single specimens of *P. mexicana*, *P. latipinna*, and *P. formosa* from natural populations using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Primers specific to 5′ and 3′ UTR regions of each of the four LWS loci were designed using genomic data from *P. reticulata* (Watson et al. 2011; GenBank accession: HM540108 and HM540107) and *Xiphophorous hellerii* (Watson et al. 2010; GenBank accession: GQ999832 and GQ999833). Primers were also designed in the 5′ and 3′ UTR regions of an RH2–1 transcript from *P. reticulata* (Hoffmann et al. 2007; GenBank accession: DQ234859). Given the high exon sequence similarity between LWS loci, we designed all primers to be locus specific (for a list of primers used and their sequences see Table S1). The LWS opsins in the Poeciliidae family have previously been identified by amino acids at position 180 of the protein, because this site is known to dramatically influence the λ_{max} of LWS opsins (Yokoyama and Radlwimmer 1998), and has been shown to differ between LWS loci in *P. reticulata* and the sister taxa in the subgenus, Micropoecilia (Ward et al. 2008). However, this nomenclature becomes confusing when describing poeciliids outside of this clade, as several species have multiple loci with the "S180" genotype (Watson et al. 2010; this study; B. A. Sandkam, unpubl. data). To avoid confusion between species, we refer to LWS loci by their location in the genome relative to one another: LWS-1 (previously A180 or S180–1), LWS-2 (previously P180), LWS-3 (previously S180 or S180–2), and LWS-R (because it is the result of a retrotransposition event)

(previously S180r) (Ward et al. 2008; Watson et al. 2010, 2011; Laver and Taylor 2011). Due to the length of LWS-2, PCR products for this gene were generated in two overlapping segments. To maintain locus specificity for these segments, we amplified each with internal and external primers. LWS-3 and the second segment of LWS-2 each had two primer sets designed for them; one specific to *P. latipinna* and one specific to *P. mexicana*.

PCR products generated from *P. formosa* DNA were cloned prior to sequencing to ensure *P. mexicana* and *P. latipinna* derived alleles were sequenced and analyzed separately. PCR products were cloned into One Shot® chemically competent TOP10 *Escherichia coli* cells using a TOPO TA Cloning® kit with pCR®2.1-TOPO® vector (Life Technologies, Burlington, ON, Canada). Plasmid DNA was isolated from overnight cultures using the QIAprep® miniprep kit (Qiagen). All sequencing was performed by Molecular Cloning Laboratories (McLab, San Francisco, CA). Sequence chromatograms were viewed and analyzed using SeqMan Pro (Lasergene 8.0; DNASTAR, Madison, WI). All sequences are available on GenBank; accession numbers JF823551–JF823570.

PHYLOGENETIC ANALYSES

All phylogenetic analyses were performed separately on the RH2–1 and LWS classes of genes, because the divergence of these opsin subfamilies predates the Poeciliidae; as a result, many gene regions within the RH2 and LWS genes are highly diverged and cannot be aligned (Rennison et al. 2011). Sequences for each group of loci were aligned using Mafft version 6.833b (Katoh et al. 2009) and edited using Se-Al version 2.0a11 (Rambaut 1996) to ensure intron–exon boundaries were consistent across LWS or RH2–1 loci for each of the three species. Best-fit models of molecular evolution were determined using MrModelTest 3.04 (Nylander 2004). Phylogenetic trees were reconstructed under Maximum likelihood (ML) using PAUP* 4.0b10 (Swofford 2003) and Bayesian methods as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Two runs using four Markov chains (three heated and one cold) were run for 10^7 generations, with trees sampled every 1000 generations for the LWS loci and RH2–1. Convergence was assessed using the SD of the split frequencies between runs, and graphically using the program Tracer (Rambaut and Drummond 2007) and AWTY (Nylander et al. 2008). ML bootstrap values and Bayesian posterior probabilities were employed to assess support. Pairwise nucleotide similarities within and between species were calculated for each locus using BLASTn (Zhang et al. 2000).

DETERMINATION OF ALLELES EXPRESSED IN A HYBRID SPECIES

Both eyes from one individual of *P. formosa* were removed and placed into RNAlater (Life Technologies) immediately after being sacrificed in an overdose of MS-222. Eyes were ground with a disposable pestle (VWR®) in 600 μl of TRIzol® reagent (Life Technologies) and homogenized in a Homogenizer Cartridge (Life Technologies). RNA was isolated using the TRIzol® Plus Purification System (Life Technologies) and subjected to an on-column PureLink® DNase treatment (Life Technologies) to ensure no DNA was present before generating cDNA. Reverse transcription was carried out using a MultiScribe Reverse Transcriptase™ kit (Life Technologies) with the addition of an RNase inhibitor (Life Technologies).

Using genomic data generated above, one PCR primer set was designed to be specific to each parental species for each locus (Table S2). The primer sets for LWS-R did not work and so the same primer set used for genomic PCR was used. PCR products of locus RH2–1 and LWS-1 were sequenced directly, resulting in "clean" single consensus sequences, without the presence of multiple peaks per base pair position in the chromatograms. A direct sequencing approach did not generate "clean" sequence for LWS-2, LWS-3, and LWS-R, therefore PCR products of these loci were cloned and sequenced following the methods described above. This approach did yield "clean" single consensus sequences. Sequences were visually compared to genomic sequences generated for each locus to confirm allele type.

BEHAVIORAL RESPONSE TO COLORED DISKS

Adult female *P. latipinna* and *P. formosa* were collected in 2011 from an introduced population (*P. latipinna* from Louisiana and Florida, [Brown 1953]; and *P. formosa* from Brownsville, Texas [Hubbs et al. 1991]) in Spring Lake at the headwaters of the San Marcos River in Hays County, Texas (29.89°N, 97.82°W) and maintained in the lab for several months before testing. Allopatric adult female *P. mexicana* were collected in Campeche, Mexico in 2002 (19.14°N, 90.50°W). Testing individuals were maintained at 25°C on a 14:10 h light:dark cycle with full spectrum UV lights (Rayon Lighting Group, Los Angeles, CA) and fed daily with Purina AquaMax 200 (Purina, Vevey, Switzerland) pellets supplemented with live brine shrimp. The spectrum and intensity of the lighting environment was not measured because all holding tanks were exposed to the same lighting.

To determine level of attraction to different colors, simultaneous choice tests were performed on individual fish by measuring the number of approaches toward and pecks at colored discs following the methodologies of Rodd et al. (2002). The testing arena used was 61 × 51 cm, filled to a depth of 15 cm and lit overhead with full spectrum fluorescent lighting (Rayon Lighting Group). Black plastic covered three sides of the arena, the fourth was covered with one-way glass to minimize disturbance from the observer. Female *P. mexicana* ($n = 17$), *P. formosa* ($n = 17$), and *P. latipinna* ($n = 15$) were tested individually. All behavioral tests were performed at Texas State University.

Four clear plastic petri dishes (diameter 9.7 cm) were randomly placed on the bottom of the tank and buried in gravel so that the lip of the dish formed a ring that could be seen by the observer and acted as an association zone with a colored disk placed in the center. Colored disks were made by painting pennies using Acrylic Colors (by Liquitex, Piscataway, NJ) either orange (Cadmium Orange Hue), yellow (Cadmium Yellow Hue), red (Naphthol Crimson), or green (Light Green). These were the same paints used by Rodd et al. 2002, which include reflectance spectra for each of the colored disks. Preliminary trials revealed that individuals were reluctant to begin foraging unless food was present; therefore, a brown food pellet (AquaMax 200, Purina, Vevey, Switzerland) was placed on top of each colored disk to elicit foraging behavior. The order in which the colored disks were arranged was randomized for each trial. A focal individual was introduced to the aquarium and allowed 10 min to begin foraging; if they did not begin foraging, they were returned to the holding tank to be tested at another time. A trial began when an individual began foraging and lasted 10 min, during which time the number of approaches (snout passes into the association zone) and number of pecks toward each colored disk were recorded. To compare the raw number of pecks and approaches toward the different colored disks, we ran repeated measures ANOVAs followed by Tukey's HSD in R 2.14.0 (R Core Development Team).

The presence of food on a disk at the start of a trial could impact the measured "preference" (pecks at and approaches toward the colored disks). However, this is unlikely to be the case because it would be expected to lead to one of two scenarios: (1) an individual could be attracted to the colored disk only to consume the food pellet—this would result in pecks/approaches toward all colors to be roughly equal, and (2) an individual could be attracted to the first color with food it comes across—this would result in the individual associating with/pecking at the first color it comes across and would be random across individuals. Neither of these two scenarios was observed; individuals had clear preferences and species were similar in preference across individuals.

Results

DATASET

At each of the five loci (four LWS and one RH2–1) sequenced in *P. formosa*, we identified two alleles. For each locus, one allele was more similar to *P. mexicana* and thus labeled the "-M" allele, and the other was more similar to *P. latipinna* and labeled the "-L" allele (for RH2–1, "-L" and *P. latipinna* are 100% identical). At every locus, the *P. formosa* "-M" allele and the *P. formosa* "-L" allele differ from one another roughly as much as the *P. mexicana* allele differs from the *P. latipinna* allele (Table 1). Assuming equal rates of molecular change, this suggests that the *P. formosa* "-M" and the *P. formosa* "-L" allele diverged at the same time as the *P. mexicana* allele and the *P. latipinna* allele, which is supported by our phylogenetic findings described below.

Table 1. Comparison of the two *P. formosa* alleles and parental species for each LWS locus and RH2–1. Percent similarity is given above the diagonal. Number of gaps is given below the diagonal. Dark gray boxes show the most similar parental sequence for each allele from *P. formosa*.

	Formosa-M	Formosa-L	Mexicana	Latipinna
LWS-1				
Formosa-M	–	98.647	99.621	98.701
Formosa-L	3	–	98.593	99.837
Mexicana	0	3	–	98.647
Latipinna	3	0	3	–
LWS-2				
Formosa-M	–	97.772	98.859	97.799
Formosa-L	25	–	98.138	99.532
Mexicana	27	4	–	96.905
Latipinna	25	12	50	–
LWS-3				
Formosa-M	–	96.346	97.310	96.346
Formosa-L	21	–	97.676	99.783
Mexicana	16	5	–	97.676
Latipinna	21	0	5	–
LWS-R				
Formosa-M	–	98.685	99.862	98.616
Formosa-L	2	–	98.824	99.931
Mexicana	0	2	–	98.754
Latipinna	2	0	2	–
RH2-1				
Formosa-M	–	99.057	99.730	99.057
Formosa-L	3	–	99.125	100.000
Mexicana	1	4	–	99.125
Latipinna	3	0	4	–

To assess functional opsin diversity, we compared amino acid sequences between species for each locus. The RH2–1 amino acid sequences were identical between *P. formosa*, *P. latipinna*, and *P. mexicana*. However, the LWS loci demonstrated considerable amino acid diversity (Table 2). As expected, all variable parental opsin haplotypes were observed in the *P. formosa* LWS repertoire with the exception of novel amino acids at one position in LWS-1, 2, and 3, and two in LWS-R. The λ_{max} of the RH2/LWS classes of opsins is primarily determined by five specific amino acid changes that occur in the region of the protein spanning the membrane (Yokoyama and Radlwimmer 1998, 2001; Yokoyama et al. 2008). Of the novel amino acids identified in *P. formosa*, none were located at any of the five sites that determine the λ_{max} of an opsin; therefore, the alleles gained from each of the parental species likely behave identically to the alleles in the parental species.

PHYLOGENETIC ANALYSIS

Phylogenetic trees inferred using ML and Bayesian methods converged upon identical topologies for both the RH2–1 and LWS

Table 2. Amino acid differences in LWS loci across species. Amino acid numbering follows that of Yokoyama (2000). Transmembrane designations indicate whether the amino acid site is located in the transmembrane domain (Y, yes; N, no), where changes in amino acids are more likely to shift spectral absorbances. The amount of within-species variation is unknown. Shading is provided to facilitate visual comparison.

	Human amino acid number																
	Locus	11	20	26	33	55	65	131	132	139	153	188	194	233	247	298	310
P. mexicana	**1**	–	–	**S**	–	–	–	**Y**	**I**	–	–	**V**	–	–	–	–	–
***P. formosa*-M**	**1**	–	–	**S**	–	–	–	**F**	**I**	–	–	**V**	–	–	–	–	–
***P. formosa*-L**	**1**	–	–	**A**	–	–	–	**F**	**T**	–	–	**I**	–	–	–	–	–
P. latipinna	**1**	–	–	**A**	–	–	–	**F**	**T**	–	–	**I**	–	–	–	–	–
P. mexicana	**2**	–	–	–	–	**A**	**I**	–	–	–	–	–	–	–	–	**A**	**L**
***P. formosa*-M**	**2**	–	–	–	–	**A**	**I**	–	–	–	–	–	–	–	–	**G**	**L**
***P. formosa*-L**	**2**	–	–	–	–	**V**	**V**	–	–	–	–	–	–	–	–	**G**	**F**
P. latipinna	**2**	–	–	–	–	**V**	**V**	–	–	–	–	–	–	–	–	**A**	**F**
P. mexicana	**3**	**Q**	–	**S**	**T**	***L***	–	–	**I**	**A**	**V**	–	**F**	**G**	–	–	–
***P. formosa*-M**	**3**	**P**	–	**S**	**T**	***L***	–	–	**I**	**A**	**V**	–	**F**	**G**	–	–	–
***P. formosa*-L**	**3**	**Q**	–	**A**	**S**	**I**	–	–	**T**	**T**	**I**	–	**Y**	**A**	–	–	–
P. latipinna	**3**	**Q**	–	**A**	**S**	**I**	–	–	**T**	**T**	**I**	–	**Y**	**A**	–	–	–
P. mexicana	**R**	–	**E**	**F**	–	–	–	–	–	–	–	–	–	–	**R**	**A**	–
***P. formosa*-M**	**R**	–	**K**	**F**	–	–	–	–	–	–	–	–	–	–	**R**	**T**	–
***P. formosa*-L**	**R**	–	**E**	**S**	–	–	–	–	–	–	–	–	–	–	**H**	**A**	–
P. latipinna	**R**	–	**E**	**S**	–	–	–	–	–	–	–	–	–	–	**H**	**A**	–
Transmembrane		**N**	**N**	**N**	**N**	**Y**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**N**	**Y**	**N**	**N**	**Y**

"–" indicates there was no variation in amino acids at that site for that locus.

opsin datasets. Support for recovered nodes was robust for all topologies inferred under both ML and Bayesian methods with no well-supported nodes differing among analyses. The fully resolved phylogenies of RH2–1 and LWS opsins are presented in Figure 1. For each locus, the "-M" and "-L" alleles of *P. formosa* group with the respective alleles from *P. mexicana* and *P. latipinna*.

DETERMINATION OF ALLELES EXPRESSED IN A HYBRID SPECIES

For four of the loci (LWS-1, LWS-3, LWS-R, and RH2–1), alleles from both parental species were expressed in the eyes of *P. formosa*. We were unable to detect the presence of LWS-2 transcripts in the eyes of *P. formosa*. This is not surprising given that recent work has shown LWS-2 to be expressed at very low levels in the closely related *P. reticulata* (Ward et al. 2008; Laver and Taylor 2011; B. A. Sandkam and F. Breden, unpubl. data). The LWS genomic organization of *P. reticulata* is identical to *P. formosa*, which likely leads to similar patterns of relative LWS expression. If *P. formosa* relative LWS expression is similar to *P. reticulata* then it is possible that LWS-2 alleles from both parents are being expressed at such low levels that we were unable to detect either of them. Based on the absence of introns, we were able to verify that each sequence originated from cDNA.

BEHAVIORAL RESPONSE TO COLORED DISKS

The proportions of pecks at and approaches toward the different colored disks are presented in Figure 2. The number of pecks made toward the green disks significantly differed between *P. mexicana* and *P. latipinna* ($P = 0.0331$), but did not differ between *P. formosa* and *P. latipinna* ($P = 0.1279$), or *P. mexicana* and *P. formosa* ($P = 0.8023$) (Table 3). These data indicate that *P. formosa* exhibits a level of attraction to green disks that is intermediate between that observed for the two parental species. The intermediate attraction of *P. formosa* was also observed in the number of approaches toward green. In contrast, *P. formosa* had a greater number of pecks toward yellow disks than *P. mexicana* ($P = 0.0334$) but not *P. latipinna* ($P = 0.2595$), and the parental species also did not differ ($P = 0.6310$). There were no significant differences between any of the species in approaches or pecks toward orange or red.

Discussion

STRONG EVIDENCE OF HSE IN *P. FORMOSA*

The phylogenetic analyses and pairwise sequence comparisons presented here clearly demonstrate that *P. formosa* possess one allele for every RH2–1 and LWS locus from each parental species, and these alleles differ from one another in amino acid sequence at several sites for the LWS opsins. Our expression analysis revealed

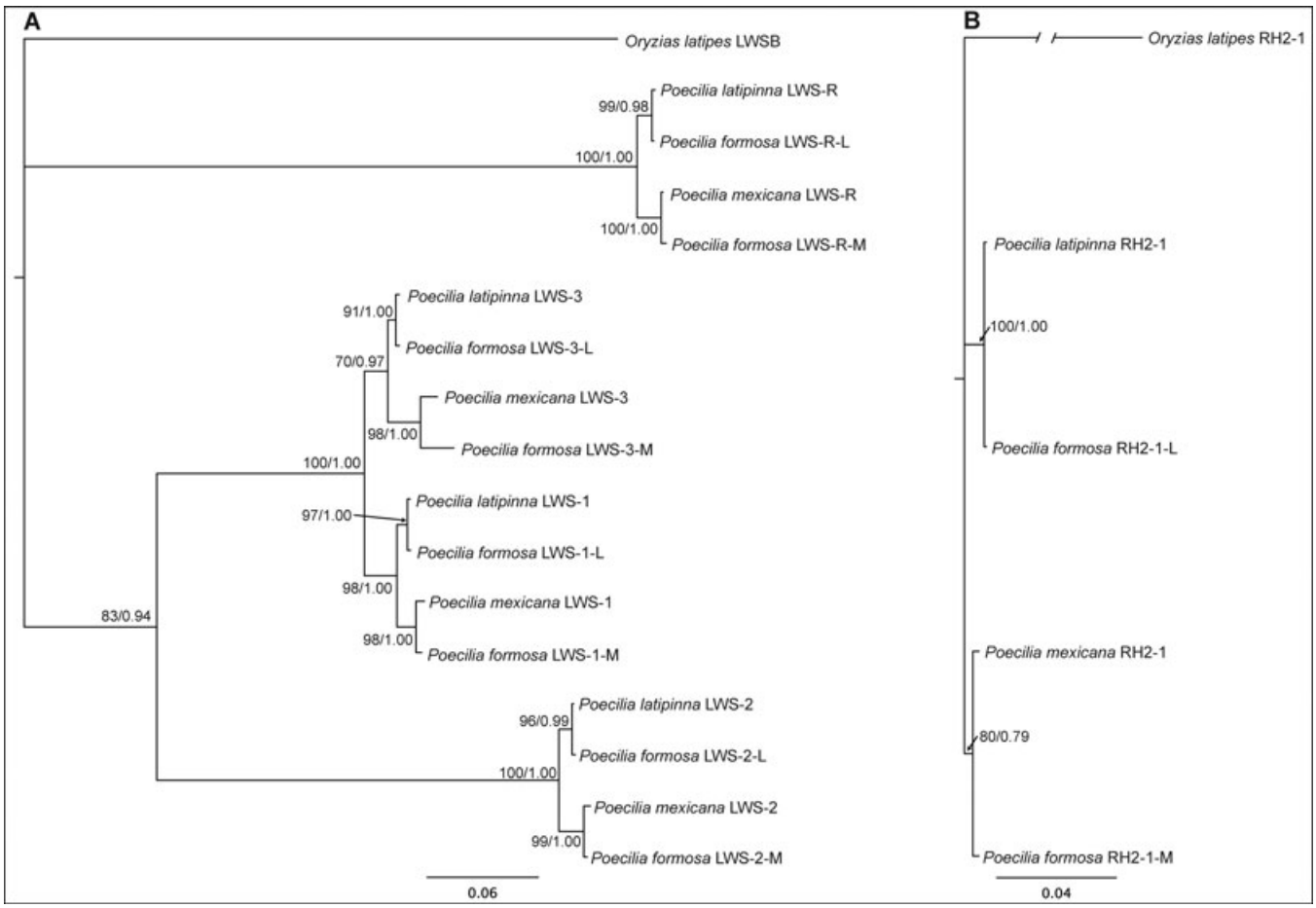


Figure 1. **Bayesian consensus phylogenies of (A) long wavelength sensitive (LWS) opsin loci, and (B) RH2–1 opsin locus in *Poecilia mexicana*, *P. latipinna*, and the two *P. formosa* alleles. Maximum likelihood bootstraps and Bayesian posterior probability values (respectively) are reported for each node.**

that *P. formosa* simultaneously express alleles from both parental species at four of the five loci we investigated (with no expression of either parental allele identified for locus LWS-2).

Microspectrophotometry (MSP), a technique used to identify the peak spectral sensitivities (λ_{max}) of a cone cell, further supports our evidence that *P. formosa* have experienced HSE in the RH2–1 and LWS opsins. Körner et al. used MSP in *P. formosa*, *P. mexicana*, and *P. latipinna* to show that the λ_{max} values of the cone cells were virtually identical across all three species except for cones tuned to long wavelength light (i.e., RH2–1/LWS). The two cone types that differed in λ_{max} absorbance between *P. mexicana* and *P. latipinna* fell in the range of the RH2–1 and LWS opsin classes (*P. mexicana*, 536.6 ± 4.7 nm and 563.0 ± 3.0 nm; *P. latipinna*, 551.2 ± 4.9 and 576.7 ± 5.9 nm) (Körner et al. 2006). Distinct spectral peaks were not able to be determined in this range for *P. formosa*, although an average peak was reported at 560.3 ± 16.1 nm. MSP is performed by directing a monochromatic light source at an individual cone cell and changing the wavelength while measuring the amount of light absorbed (Loew and Lythgoe 1978). Specific absorbance peaks for cone cells are determined by averaging groups of similar absorbances; this procedure makes assigning cone types to groups difficult when cones have similar λ_{max} values. Given that *P. formosa* have opsins that closely match the amino acid sequence of each of their parental species' opsins (Table 2), and that our expression data show they are all expressed, the inability to determine a spectral peak in *P. formosa* is most likely the result of producing all of the parental opsin types in their cone cells. Because the parental species have cones with λ_{max} values at 536.6 ± 4.7 and 563.0 ± 3.0 nm (*P. mexicana*) and 551.2 ± 5.9 and 576.7 ± 5.9 nm (*P. latipinna*), it is possible that the 560.3 ± 16.1 nm range in *P. formosa* is actually four cone types with λ_{max} values at 536, 551, 563, and 576 nm. If one is to consider all *P. mexicana* and *P. latipinna* RH2–1/LWS cone types at once, the distance between the λ_{max} plus the SD of one cone and λ_{max} minus SD of the next highest cone is smaller than the SDs themselves; 4.0, 3.1, 4.8 nm between 536.6, 551.2, 563.0, and 576.7 nm, respectively. Therefore, if all cone types from the parental species were present in the same eye, the

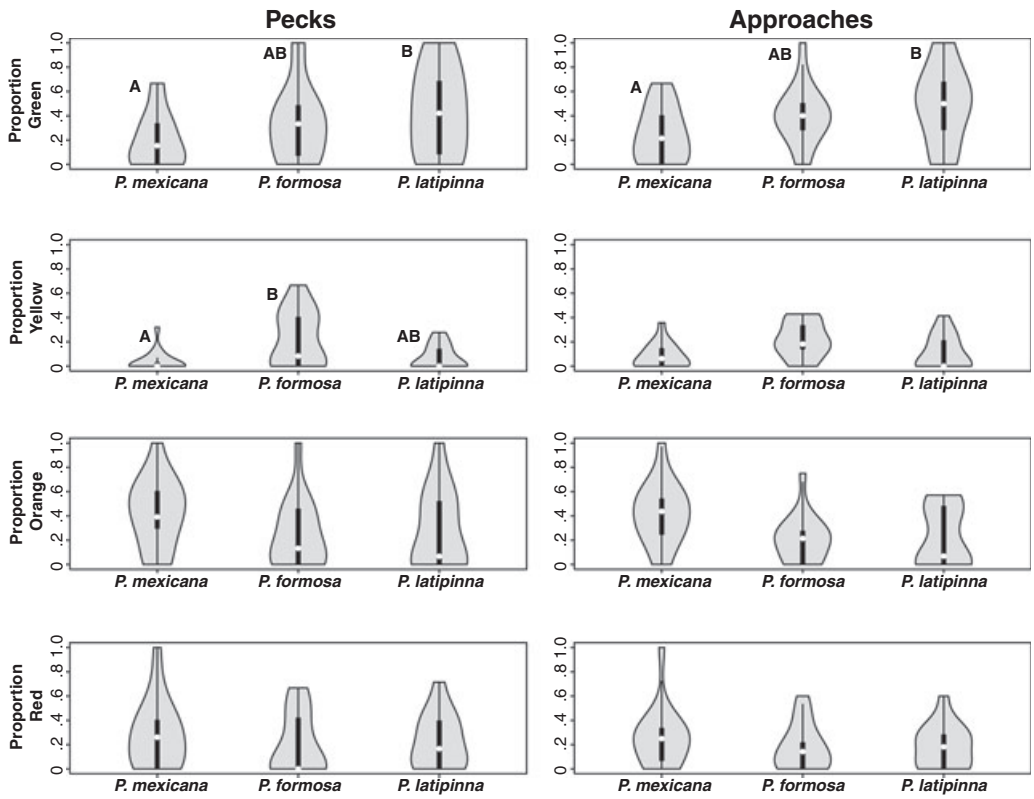


Figure 2. Violin plots (Hintze and Nelson 1998) showing the proportion of pecks and approaches made toward each colored disk for the hybrid *P. formosa* and the parental species *P. mexicana*, and *P. latipinna*. Violin plots contain a box plot (white circles denote mean) surrounded by a gray kernel density plot, which shows the distribution of the data followed by smoothing to facilitate visual comparison across plots. Letters denote groups that differ significantly (Tukey's HSD, $P < 0.05$).

Table 3. Tukey's HSD comparing the hybrid *P. formosa* and parental species, *P. mexicana* and *P. latipinna*, in the number of pecks and approaches made toward colored disks. Bold values indicate significance at $P < 0.05$.

		Pecks			Approaches		
		P-value	*F*-value	df	*P*-value	*F*-value	df
Green	*P. latipinna*×*P. formosa*	0.1279	3.625	2	0.8792	3.920	2
	P. mexicana×*P. formosa*	0.8023	3.625	2	0.0856	3.920	2
	P. mexicana×*P. latipinna*	**0.0331**	3.625	2	**0.0334**	3.920	2
Yellow	*P. latipinna*×*P. formosa*	0.2595	3.436	2	0.4062	1.860	2
	P. mexicana×*P. formosa*	**0.0334**	3.436	2	0.1565	1.860	2
	P. mexicana×*P. latipinna*	0.6310	3.436	2	0.8594	1.860	2
Orange	*P. latipinna*×*P. formosa*	0.9969	1.323	2	0.5459	1.815	2
	P. mexicana×*P. formosa*	0.3567	1.323	2	0.6578	1.815	2
	P. mexicana×*P. latipinna*	0.3411	1.323	2	0.1488	1.815	2
Red	*P. latipinna*×*P. formosa*	0.4646	0.757	2	0.9766	0.521	2
	P. mexicana×*P. formosa*	0.6756	0.757	2	0.7205	0.521	2
	P. mexicana×*P. latipinna*	0.9274	0.757	2	0.6075	0.521	2

resulting λ_{max} values would form a near-continuous distribution, which would be indistinguishable from one cone cell type with a λ_{max} that has a high SD, such as that reported for *P. formosa*. Although it is clear that the additional opsin alleles are being used, it is also possible that *P. formosa* are expressing two opsin alleles per cone cell (functional implications will be discussed below).

Another possibility is that alleles of either *P. latipinna* or *P. mexicana* are acting in a dominant/recessive manner.

Having dominant alleles from one parental species would result in *P. formosa* possessing a sensory system that matches one of the parental species. The data generated from the MSP study by Körner et al. (2006) clearly show that *P. latipinna*, *P. mexicana*, and *P. formosa* have dramatically different visual systems in the range of the LWS opsins, and thus that the LWS opsins are not acting in a dominant/recessive manner. Therefore, the LWS opsin alleles from one locus are either expressed in different cone cells (as discussed above), or in the same cone cell, and are acting in a codominant fashion. Either way, the RH2–1/LWS opsin repertoire is clearly expanded in *P. formosa* as a result of the hybridization of *P. mexicana* and *P. latipinna*. Thus, our results provide strong support for the HSE hypothesis.

WHAT DOES HSE MEAN FOR *P. FORMOSA* VISION?

Vision is the detection of different wavelengths of light, whereas color vision is the discrimination of different wavelengths of light. Adding photoreceptor pigments can expand the detectable spectral window and/or expand the ability to discriminate wavelengths (i.e., color discrimination) (Jacobs et al. 1999). Color discrimination is accomplished by comparing the signals from cone cells with different λ_{max}. In normal color vision each cone cell expresses only one opsin (Hagstrom et al. 2000), thereby maximally detecting only one wavelength of light. Because *P. formosa* are expressing two alleles at the same locus, it is possible that their cone cells have two wavelengths that they 'maximally' detect. Some New World primates are heterozygous for MWS/LWS opsin alleles but do not have this "problem" because their opsin locus is on the X chromosome, therefore only one allele is expressed per cell due to X-inactivation (Jacobs 1998). Mapping data and synteny comparisons of LWS opsin gene regions between the *P. reticulata* and *Oryzias latipes* genomes suggest that *P. reticulata* LWS opsin genes are not sex-linked (Tripathi et al. 2009; Watson et al. 2011). Given the close similarity between species in Poeciliidae (Breden et al. 1999; Hamilton 2001), it is likely that this is also true for *P. formosa*. However, it is not known whether multiple opsin genes are expressed in individual cone cells in fish retinas, or if some form of allelic exclusion exists.

Two studies by Jacobs et al. (1999, 2007) highlight possible implications of an expanded opsin repertoire. Jacobs et al. (2007) created a line of transgenic mice in which the cone cells either expressed an MWS opsin or an LWS opsin. The mice had an expanded range of wavelengths they could detect and were able to differentiate additional wavelengths of light. However, Jacobs et al. (1999) also created a line of transgenic mice with MWS and LWS opsins but had to coexpress them in individual cone cells. The mice could detect an expanded range of wavelengths but were unable to differentiate wavelengths of light (i.e., see color). It is unknown if HSE in *P. formosa* has resulted in an increased ability to differentiate wavelengths or only an expanded visual range. Our behavior experiments suggest that *P. formosa* still have the ability to differentiate wavelengths of light. The extent that *P. formosa* pecked at/approached painted disks differed by color in a manner similar to the parental species that are homozygous at opsin loci (Fig. 2). This pattern held except for attraction to green (*P. formosa* is intermediate to the parental species) and attraction to yellow (*P. formosa* is more attracted than either parental species). Thus, the combination of parental opsin loci through hybridization has led to functional differences in the tuning of the sensory system of *P. formosa*.

UNANTICIPATED PECKING BEHAVIOR

Using MSP, both *P. mexicana* and *P. latipinna* have been shown to possess two classes of cone cells that maximally detect light in the range of green, yellow, red, and orange. While *P. mexicana* cones detect wavelengths around 536 and 563 nm, cones of *P. latipinna* detect wavelengths around 551 and 576 nm (Körner et al. 2006). Both cone classes in *P. mexicana* maximally detect shorter wavelengths of light than those of *P. latipinna*. Because *P. mexicana* are better tuned to detect shorter wavelengths than *P. latipinna*, we hypothesized that *P. mexicana* would peck at colored disks that reflect shorter wavelengths of light more than *P. latipinna.* The yellow- and green-colored disks used in our behavioral trials reflect shorter wavelengths than the red- and orange-colored disks (Rodd et al. 2002). Therefore, we predicted that *P. mexicana* would peck at green and yellow disks more than *P. latipinna*, whereas *P. formosa* would be intermediate to the two parental species because it has opsins from both. Surprisingly, we found that *P. mexicana* pecked at the green disks less than *P. latipinna*, whereas *P. formosa* behaved as predicted (intermediate to the parental species). The unexpected switch in pecking behavior between *P. mexicana* and *P. latipinna* is interesting and may be an outcome of a female preference for the green-blue coloring of large male sailfin mollies but further exploration by the field of behavioral ecology is needed to test this hypothesis. Despite the unanticipated behavior of the parental species, it should be noted that *P. formosa* did behave as predicted; pecking the green disk at a proportion that was intermediate to the parental species. Clearly, *P. formosa* has inherited opsin alleles from both *P. mexicana* and *P. latipinna*, which has influenced the sensory system and thus behavior of this hybrid species.

FITNESS IMPLICATIONS OF HSE

Our data show that *P. formosa* have received the RH2–1 and LWS opsin repertoire of both parental species. This raises the question of whether this expanded repertoire has impacts on *P. formosa* fitness. There have been at least 840,000 generations since the hybridization event that lead to the formation of *P. formosa* (Stöck et al. 2010) and yet opsin amino acid sequences remain almost identical to their parental sequences (Table 2); this is possibly due

to selection for the maintenance of functional opsins with the same λ_{max} as their parental species (although slow neutral evolution is still a possibility [Schartl et al. 1991]). We therefore suggest that tests of the HSE in systems where sensory stimuli influence fitness through foraging or mating decisions may prove useful for understanding the importance of hybridization in expanding sensory system repertoires (such as crickets [Shaw and Herlihy 2000], flies [Schwarz et al. 2005; Mallet 2007], cichlids [Carleton and Kocher 2001], and butterflies [Awata et al. 2009]).

Conclusion

Taken together our results clearly show that hybrid species can have a different sensory repertoire than either of their parental species, because they share sensory genetic variation with both parents. Bringing together sensory allelic variation from both parental species results in an expanded sensory system in one generation that is much faster than the more traditional models of duplication and divergence. We have termed this phenomenon "hybrid-sensory expansion" (HSE) and propose that it could help explain many differences between hybrids and their parental species. Providing hybrids with large sensory repertoires made up of alleles that have experienced selection in two different species could result in different outcomes depending upon the selective environment of the hybrid. Given a selective environment that is similar to one or both of the parental species, the hybrid may exhibit the same tuning of the sensory repertoire to either or both parental species. However, it is also possible that divergent selection could act on the allelic diversity within the hybrid to favor differentiation of the sensory system, resulting in different sensory alleles from the parental species.

In some cases, hybridization may thus be an important driver of the diversification of sensory systems via repertoire expansion and this process likely catalyzes sensory diversification among other organisms and in the context of other sensory systems.

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Supporting Information

The following supporting information is available for this article:

Table S1. Primers used for creating PCR products and sequencing.

Table S2. Primers used for creating cDNA products.

Supporting Information may be found in the online version of this article.

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