Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2012) **11:** 222–229 doi: 10.1111/j.1601-183X.2011.00742.x

The mate choice brain: comparing gene profiles between female choice and male coercive poeciliids

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Genes that mediate mate preferences potentially play a key role in promoting and maintaining biological diversity. In this study, we compare mate preference behavior in two related poeciliid fishes with contrasting behavioral phenotypes and relate these behavioral differences to gene profiles in the brain. Results reveal that one poeciliid fish, the Northern swordtail, exhibits robust mate preference as compared to the Western mosquitofish, which utilizes a coercive mating system. Female swordtails display no significant difference in association time between male- and female-exposure trials, whereas female mosquitofish spend significantly less time associating with males relative to females. Furthermore, the preference strength for large males is significantly lower in female mosquitofish relative to swordtails. We then examine expression of three candidate genes previously shown to be associated with mate preference behavior in female swordtails and linked to neural plasticity in other vertebrates: *neuroserpin* (NS), *neuroligin-3* (NLG-3) and *N-methyl-D-aspartate receptor* (NMDA-R). Whole brain gene expression patterns reveal that two genes (NS and NLG-3) are positively associated with mate preference behavior in female swordtails, a pattern opposing that of the mosquitofish. In mosquitofish females, these genes are downregulated when females express biases toward males yet are elevated in association with total motor activity patterns under asocial conditions, suggesting that the presence of males in mosquitofish species may inhibit expression of these genes. Both gene expression and female behavioral responses to males exhibit opposing patterns between these species, suggesting that this genetic pathway may potentially act as a substrate for the evolution of mate preference behavior.

Keywords: Candidate genes, coercive mating, mate choice, *neuroligin-3*, *neuroserpin*, *NMDA receptor*, poeciliid

Received 13 May 2011, revised 26 August 2011 and 4 October 2011, accepted for publication 12 October 2011

Introduction

Female mate choice is a social behavior that exhibits considerable variation both within and between species. Identifying and understanding genes underlying female mate preference provides insight into the evolution of preference behavior. Multifactorial approaches are commonly used to explore genomic regulation of many social behaviors, including aggression, courtship, mating and division of labor (Aubin-Horth *et al*. 2005; Ellis & Carney 2011; Filby *et al*. 2010; Mukai *et al*. 2009; Whitfield *et al*. 2003). Although few studies have assessed the various molecular pathways that underlie female mate preference behavior, one study identified candidate genes uniquely regulated under mate preference conditions in a teleost: the Northern swordtail (*Xiphophorus nigrensis*; Cummings *et al*. 2008). In this study, we explore how three of these candidate genes *neuroserpin* (NS), *neuroligin-3* (NLG-3), and *N-methyl-D-aspartate receptor* (NMDA-R; partial NR1 subunit; see Table S1 for gene nomenclature) are modulated during mate choice in species with vastly different female responses to males. These genes play a clear role in regulating social behavior in other taxa (Biswas *et al*. 2010; Madani *et al*. 2003; Radyushkin *et al*. 2009) and are functionally related in that they regulate synaptic plasticity, an event linked to learning and memory. Neural plasticity-related experiences such as learning-based mate preferences are observed across a wide variety of taxa (ten Cate & Rowe 2007 for review), including the Northern swordtail in which experience and age-dependent preferences have been shown (Wong *et al*. 2011). Hence, exploring the role of a molecular pathway underlying neural plasticity-related events may provide insight into molecular substrates that may serve as targets for selective processes. We compare the whole brain expression profiles of three mate preference candidate genes between two related poeciliid species with contrasting behavioral phenotypes to better understand the genomic pathways regulating this critical behavior in sexual selection.

Poeciliids are live-bearing, freshwater fish that are a classic system in which to test questions pertaining to sexual selection via female mate choice. However, close to half of all poeciliid species reproduce in a coercive mating system in which the female exerts little or no mate preference (Bisazza 1993). We chose two poeciliids with contrasting mating systems in which females display substantially different responses to visual stimuli in males. Visual cues are sufficient to elicit preference response in a number of different poeciliid species (Basolo 1990; Rosenthal & Evans 1998; Fig. 1

Figure 1: Photographs of two male poeciliid fishes. (a) Northern swordtail (*Xiphophorus nigrensis*) and (b) Western mosquitofish (*Gambusia affinis*). The males of these species display variation in ornamentation and courtship behavior. Photos courtesy of Erich Schlegel.

for photographs), and thus visual cues should capture a significant portion of whole brain gene responses. We compare gene expression profiles in Northern swordtail females that exhibit substantially different preference behavior compared with female Western mosquitofish (*Gambusia affinis*). Some mosquitofish species display coercive mating and exhibit little or no female mate choice (Bisazza & Marin 1991, 1995). Our aim is to explore whether three functionally related preference candidate genes exhibit differential expression profiles between related species with contrasting mating systems. Such comparative studies are essential for understanding the evolution of genes that may mediate a key female behavior that facilitates biological diversity and help to clarify the evolutionary connections between genes and behavior.

Methods

Behavior

In some species of mosquitofish females exhibit seasonal ovarian recrudescence (Edwards *et al*. 2010), and preliminary studies reveal seasonal changes in receptive behavior in our focal species, the Western mosquitofish. Therefore, all behavioral tests in these females were conducted in July–August of 2010 when ovaries are recrudesced (Edwards *et al*. 2010). In contrast, female swordtails do not exhibit changes in receptivity as a consequence of changes in season (Morris & Ryan 1992) or reproductive state (Ramsey *et al*. 2011). These females are reproductively active all year (Morris & Ryan 1992). Therefore, these behavioral tests were conducted in October–December 2008 and January–May 2009. Size of the focal female mosquitofish and swordtail ranged from 41 to 24.5 mm and from 34 to 23 mm, respectively. To ensure social responses in poeciliid fish, including swordtails and mosquitofish, pre-isolation from males is commonly used before behavioral observations (Hughes 1995). As in Cummings *et al*. (2008), female swordtails were socially isolated for at least 2 weeks before starting behavioral observations. We also isolated female mosquitofish for approximately 2 weeks before behavioral observations. Social isolation allowed us to limit social experiences, including sexual experiences, immediately before behavioral observation. However, the full suite of mate preference experiences of these females during development is unknown.

Female mosquitofish and swordtails were randomly assigned to a group in which the female was exposed to: large and small male pair ($n = 40$ mosquitofish; $n = 14$ swordtails), two size matched females ($n = 20$ mosquitofish; $n = 7$ swordtails) or empty experimental compartments (i.e. no social exposure; $n = 18$ mosquitofish). Fewer swordtail than mosquitofish were used in these experiments because of permit restrictions. The swordtail females were from an ongoing experiment in which only non-foraging females were analyzed. Of the 14 swordtail females, eight had experienced a pretest with a large/small male pair 5 days before trials as part of a separate experiment. We compared mosquitofish behavior in an asocial environment with previously reported swordtail behavior in an asocial environment (Cummings *et al*. 2008) as a point of reference. The standard length (SL) of stimulus males that were considered large ranged from 29.6 to 37 mm in mosquitofish and from 34.6 to 44.4 mm in swordtails, whereas the SL of stimulus males that are considered small ranged within 18.9–26.2 mm in mosquitofish and 20.7–25.7 mm in swordtails. The average difference in male SL was 6.97 mm for mosquitofish and 14.3 mm for swordtails. Experimental tank setup and mate preference behavior trials with female swordtails and mosquitofish were as described in Cummings *et al*. (2008). Briefly, behavior tests utilized a dichotomous choice design in which fish used as stimuli were placed behind Plexiglas dividers in the end zone compartments, whereas the focal fish was placed in the center compartment. The center compartment was divided into three zones: two association zones and a central neutral zone. A trial began with the female in the neutral zone for a 5-min acclimation period followed by two 15-min observation periods, in which we recorded the time spent in the association zone and measures of activity (i.e. number of transits between association zone and neutral zone). Because female mosquitofish do not express receptivity displays, comparisons to female swordtail behaviors were limited to association time and overall activity measurements only. We calculated total association time (total time spent in both association zones), proportion of time spent with either the large or small male, and association bias (time spent in association with one individual/total association time). After the first 15-min observation period, fish used as stimuli were then switched to control for side bias and behavior was recorded for an additional 15 min. It is important to note that these studies have eliminated multimodal communication by allowing female access to only visual cues in these tests.

Gene profiling

Immediately following the completion of the behavioral observations females were sacrificed and brain tissue was extracted from a subset of mosquitofish females. Sample sizes for extracted brain tissue are as follows: male exposed ($n = 10$ mosquitofish), female exposed ($n = 10$ mosquitofish) or no social exposure ($n = 8$ mosquitofish). Tissue was stored in RNAlater (Applied Biosystems, Carlsbad, CA, USA) at −80C until RNA extraction. All procedures were approved by IACUC at the University of Texas at Austin (protocol number: AUP-2010-00148). Gene sequencing, cloning and qPCR were conducted as described in Cummings *et al*. (2008; see Table S1 for list of primers, parameters and gene accession numbers). Briefly, brain tissue was homogenized and RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA, USA). Extracted RNA was DNase-treated using turbo DNA-free kit (Applied Biosystems) before cDNA synthesis. Reverse transcription of cDNA was done using Superscript First-Strand Synthesis (Invitrogen). The cDNA synthesis was primed with both oligo-dT and random hexamers using a modified Invitrogen protocol for qPCR template. Cloning and qPCR primers were designed using MacVector software program, and primers were purchased from International DNA Technologies (IDT, Alville, IA, USA). The qPCR was conducted using SYBR green detection on an ABI prism 7900 qPCR machine (Applied Biosystems) with each sample run in triplicate. The qPCR run results were first analyzed using the Applied Biosystems Sequence Detection System software (SDS v. 2.2.1), and gene expression levels were normalized to cDNA input quantities as measured by a RiboGreen RNA quantification assay (Aubin-Horth *et al*. 2005; Cummings *et al*. 2008; Hashimoto *et al*. 2004). We used the Ribogreen assay [Quant-iT RiboGreen RNA reagent (Molecular Probes, Invitrogen)] for precise determinations of

cDNA concentrations and to therefore eliminate housekeeping gene comparisons, which have been shown repeatedly to be variable in expression across experimental conditions (Bustin 2002; Hashimoto *et al*. 2004). Previous experiments showed that RiboGreen reagent measured single-stranded RNA and cDNA with equal effectiveness (see supplementary table 2 in Cummings *et al*. 2008). In Cummings *et al*. (2008), we quantified template before (DNase-treated total RNA) and after (RNase-treated cDNA) the RT reaction. Results revealed a 0.958 correlation between the two measurements. Because cDNA quantification better reflects the actual target input into each well in the qPCR procedure, we chose to use cDNA estimates in our previous study as well as the study presented here. The estimated quantity from the qPCR results were averaged across the three replicates. We conducted a linear regression using the mean quantity for each subject (as measured by qPCR) and the initial cDNA used in the assay (as measured by RiboGreen). The residuals of this regression produced values that normalized input quantity/individual and therefore, these residual values were used in our statistical analyses.

Gene sequencing, cloning and qPCR were conducted for three mate preference candidate genes and one negative control gene. NS, NLG-3 and NMDA-R were uniquely expressed in mate choice conditions relative to other social environments in a previous microarray experiment conducted in swordtails (Cummings *et al*. 2008; see table S1). In addition, two of these genes (NS and NLG-3) exhibited significant positive correlations between whole brain expression and female mate preference behavior in swordtails. Furthermore, NS, NLG-3 and NMDA-R are functionally related and involved in social behavior in other taxa. We selected these three specifically because of their documented roles in synaptic plasticity and long-term potentiation (LTP) to explore the relationship between preference behavior and a putative synaptic plasticity pathway. We also examined a negative 'control' gene, Early B-cell associated zinc finger transcription factor (Early B). Early B was chosen as a negative control gene because our previous microarray study revealed that Early B was associated with female association environments and was not differentially expressed in male-exposed environments (Cummings *et al*. 2008; Table S1). Therefore, Early B serves as a control for genes not associated with mate choice conditions. Our sample sizes for the control gene analysis ($n = 8$) were smaller in male-exposed female mosquitofish as compared to the experimental genes because of a lack of remaining cDNA in two of the mosquitofish females.

Statistics

Behavior

A two-way analysis of variance (ANOVA) compared the time spent in the association zones as a consequence of male size (i.e. large/small males) and as a consequence of species (i.e. mosquitofish vs. swordtail) as well as the interaction between these factors. A one-way ANOVA with Bonferroni *post hoc* comparisons compared the total time that mosquitofish females spent in the association zones in the male, female and asocial-exposed groups. A student's *t* test was used to compare the total time swordtail females spent in the association zones in the male and female-exposed groups.

Gene expression

A Shapiro–Wilks test revealed that some behavioral measurements (i.e. association bias) and gene residuals were not normally distributed. However, there were no differences in the results whether we used (1) transformed normalized data, (2) non-transformed data in a parametric test or (3) nonparametric Spearman's correlation. Because results were consistent with all three methods, we used linear regression on nontransformed data to examine the relationship between association bias during mate preference trials and the expression of NS, NLG-3 and NMDA-R in swordtails and mosquitofish. However, in the NMDA-R data and Early B data only, there were extreme outliers in the male-exposed subjects in both species. We removed these subjects. The adjusted sample sizes for NMDA-R was $n = 12$ and $n = 8$ for swordtails and mosquitofish, respectively, and $n = 7$ for Early B measurements in mosquitofish. Linear regression was also used to examine the relationship between gene expression and total transits. We used a Benjamini–Hochberg test (Benjamini *et al*. 2001) to control our false discovery rate (FDR) due to multiple comparisons. In addition, we used analysis of covariance (ANCOVA) to identify any interaction between behavior–gene relationships across the two species. This analysis allowed us to determine whether the slopes of the regression line were significantly different between the species in male-exposed females.

Results

Behavior

Swordtail females spent equivalent amounts of time associating with males and females (Fig. 2a; $t = 1.01$; df = 19; $P = 0.32$). This is in direct contrast with the pattern observed with mosquitofish females. In mosquitofish, females differed in the total time spent in the association zones between male, female and asocial-exposed groups (Fig. 2a; $F = 9.46$; df = 2, 77; $P = 0.0002$). Bonferroni *post hoc* comparisons revealed that females spend significantly less time with males as compared to females ($P < 0.001$), and less time in the association zones when males were present as when the stimulus ends were empty (asocial control; $P = 0.02$). The results of a two-way ANOVA revealed that female swordtails spent significantly more time in the association zone with males as compared to mosquitofish (main effect of species: $F = 10.16$; df = 1, 107; $P = 0.0019$; Fig. 2b). Despite species level differences in socializing across different social contexts, in the male-exposed conditions both species spent significantly more time with the large male as compared with the small male (main effect of size: $F = 24.95$; df = 1, 107; $P = 0.0001$; Fig. 2b). Furthermore, because the magnitude of the size differences between stimulus males was substantially different between swordtails and mosquitofish, we explored whether these differences predicted variation in association biases in each species. However, the magnitude in the size difference between large and small males did not significantly predict variation in female association bias in swordtails (Pearson correlation coefficient, $r = 0.04$; $P = 0.89$) nor in female mosquitofish ($r = -0.20$; $P = 0.23$). Finally, there was a trend toward a significant interaction between species and the proportion of time spent with large/small male (interaction: $F = 3.21$; df = 1, 107; $P = 0.076$; Fig. 2b). For a point of reference, we conducted a two-way ANOVA to compare the behavior of these female mosquitofish to female swordtail behavior as reported in Cummings *et al*. (2008). This analysis further supports the results described above: female swordtails associate with males significantly more than female mosquitofish (main effect of species: $F = 6.09$; df = 1, 93; $P = 0.015$) and both species spend significantly more time with large males as compared with small males (main effect of size: $F = 28.8$; df = 1, 93; $P < 0.0001$). However, there is a significant interaction between species and time spent with large males (interaction: $F = 9.25$; df = 1, 93; $P = 0.003$), which reveals that female swordtails spend a significantly greater proportion of their time associating with large males as compared to female mosquitofish.

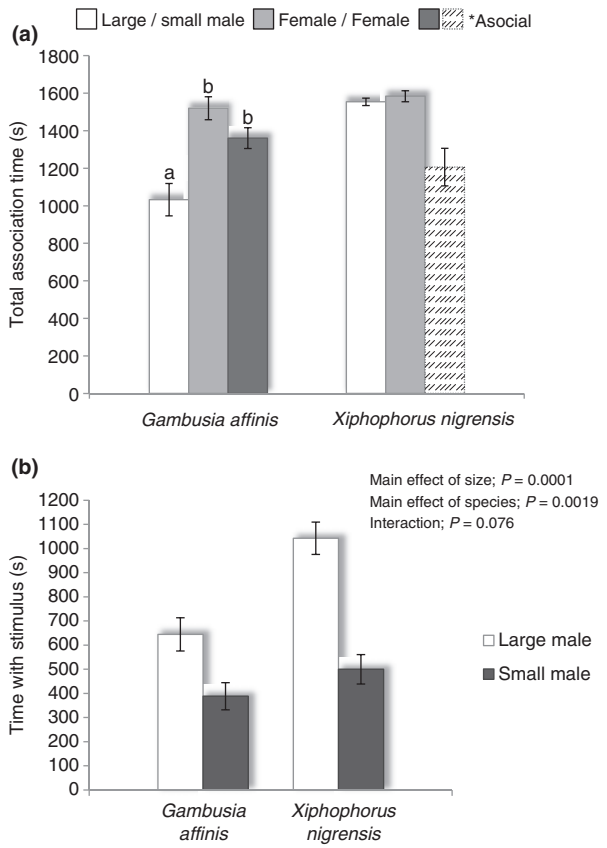

Figure 2: Behavioral comparison between species. (a) Comparison of the total association time in male-exposed, female-exposed or empty chamber-exposed female mosquitofish (i.e. asocial exposure). Female swordtails were either male-exposed or female-exposed and therefore, the asocial-exposure group represented here for swordtails is data reported by Cummings *et al*. 2008; these data were not included in the statistical analysis but are represented here as a point of reference*. (b) Comparison of the amount of time female mosquitofish and female swordtails spend in association with large conspecific males as compared to small conspecific males. The time spent with males is also compared between the species.

Gene expression

Similar to the pattern observed in a previous study (Cummings *et al*. 2008), female swordtails in this study showed a positive relationship between association bias and the expression of NS and NLG-3 (Fig. 3a–b) in females exposed to large and small males. There was a significant positive relationship between NS and association bias in these male-exposed female swordtails ($P = 0.013$; FDR $\alpha = 0.033$; Fig. 3a), however, the positive relationship between association bias and NLG-3 was nearly significant at the 0.05 level ($P = 0.058$; Fig. 3b). Association bias was significantly negatively correlated with NMDA-R in these swordtail females ($P = 0.008$; FDR $\alpha = 0.016$; Fig. 3c). On the contrary, in female mosquitofish exposed to the large and small male pairing, there was a consistent negative relationship between association bias and the expression of NS and NLG-3, but no relationship with NMDA-R (Fig. 3d–f). The negative relationship between NLG-3 and association bias was significant at the 0.05 level in mosquitofish ($P = 0.049$; Fig. 3e), although this did not meet the FDR requirements ($\alpha = 0.016$). Variation in association biases nearly significantly predicted NS expression at the 0.05 α level (NS; $P = 0.054$; Fig. 3d) but not NMDA-R expression ($P = 0.67$; Fig. 3f).

An ANCOVA revealed significantly different relationships between NS expression in male-exposed females due to species (df = 20; $t = -2.41$; $P = 0.025$) and due to association bias (df = 20; $t = -3.16$; $P = 0.004$), as well as a significant interaction between the slopes of these lines (df = 20; $t = 2.43$; $P = 0.024$). Similarly, the relationship between NLG-3 expression was significantly different due to species (df = 20; $t = -2.438$; $P = 0.024$) and due to association bias (df = 20; $t = -3.22$; $P = 0.004$), with a significant interaction (df = 20; $t = 2.46$; $P = 0.023$). There was no relationship in NMDA-R expression due to species (df = 20; $t = 0.095$; $P = 0.926$) or due to association bias (df = 20; $t = .111$; $P = 0.913$) and no interaction between them (df = 20; $t = -.116$; $P = 0.909$).

In contrast to the male-exposed conditions, both swordtail and mosquitofish females in the female-exposed conditions exhibited no significant differential gene expression with association bias or total activity (Table 1), and no correlations with association bias in the asocial condition in the mosquitofish (Table 1). In female mosquitofish, there was a significant relationship between total activity and NS and NLG-3 under asocial conditions ($P = 0.04$ for both genes) and a non-significant trend between total activity and NMDA-R ($P = 0.07$). However, neither of these relationships between NS or NLG-3 and total activity met the FDR requirements ($\alpha = 0.033$).

Our negative control gene (Early B) exhibited no expression patterns with female preference behavior in either species. In female mosquitofish and swordtails, mate preference behavior did not significantly predict the expression of Early B in any of the social conditions (mosquitofish: LS: $r = 0.47$; $P = 0.29$; FF: $r = 0.048$; $P = 0.89$; AS: $r = 0.39$; $P = 0.33$; swordtails: LS: $r = 0.11$; $P = 0.69$; FF: $r = 0.27$; $P = 0.55$).

Discussion

We chose two poeciliids with contrasting mating systems in which females display substantially different responses to potential mates. Swordtail females exhibit robust preferences for male characteristics such as size and ornamentation (Ryan & Rosenthal 2001). Large males commonly have conspicuous ornaments and perform courtship displays (Fig. 1). Female swordtails exhibit receptive responses to courtship behaviors (Cummings & Mollaghan 2006). On the other hand, mosquitofish utilize a coercive mating system in which the males force or sneak copulations, display little or no courtship and lack conspicuous ornaments (Fig. 1; Bisazza 1993; Farr 1989). Gravid female mosquitofish lack receptive behaviors and avoid sexually active males (Bisazza *et al*. 2001; McPeek 1992). In this study, we show that the two species associate with males in a dramatically different

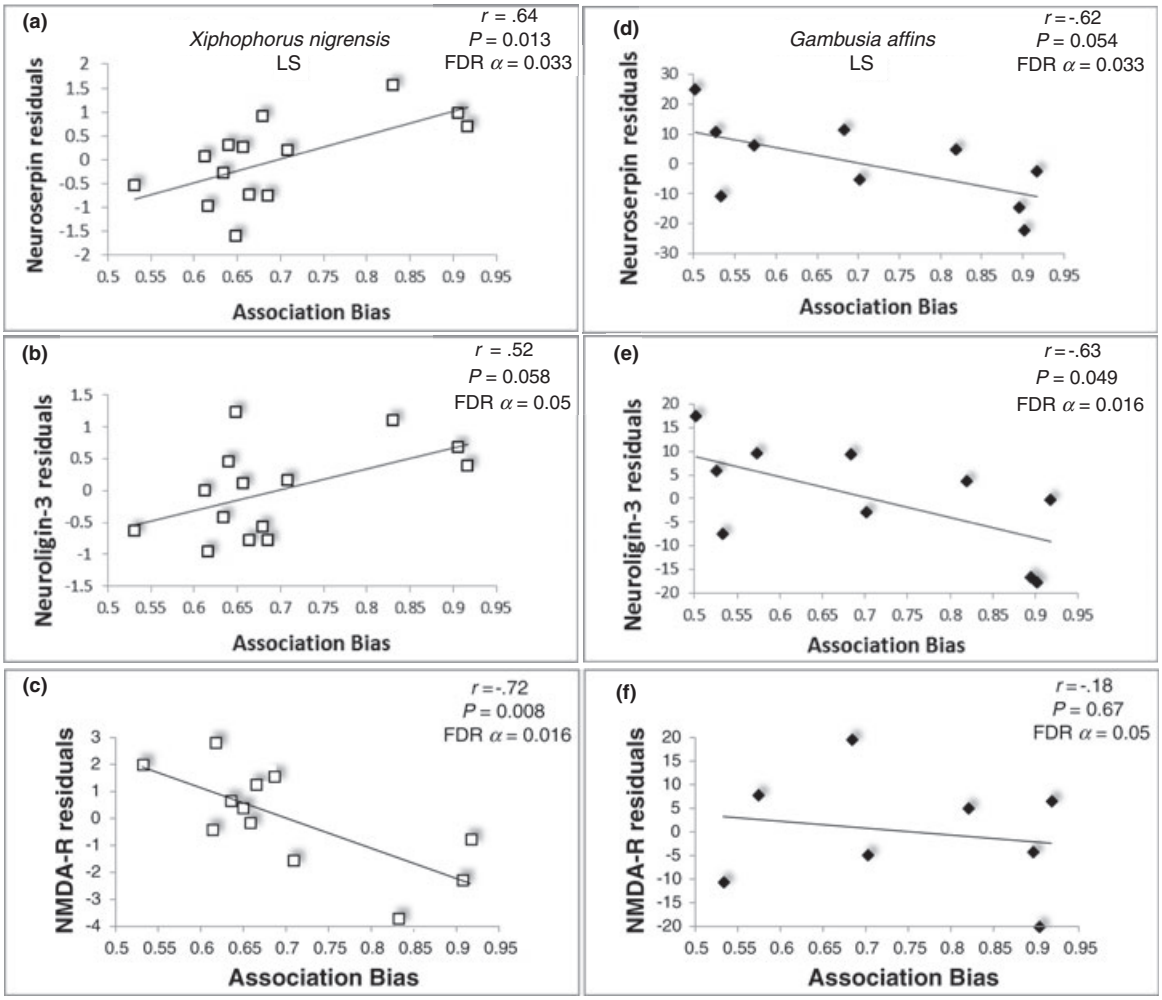

Figure 3: The relationship between gene expression and association bias in females exposed to males in two species of poeciliid fish. The relationship between male-exposed female Northern swordtails and gene expression for (a) *Neuroserpin* (NS); (b) *Neuroligin-3* (NLG-3) and; (c) *N-methyl-D-aspartate receptor* (NMDA-R). The relationship between male-exposed female Western mosquitofish and gene expression for NS (d), NLG-3 (e) and NMDA-R (f). Planned post-hoc comparisons using an ANCOVA revealed a significant interaction between the species in the NS-behavior relationship (df = 20; $t = 2.43$; $P = 0.024$) as well NLG-3-behavior relationship (df = 20; $t = 2.46$; $P = 0.023$). There was no significant interaction between NMDA-R and behavior (df = 20; $t = .118$; $P = 0.908$).

fashion. First, female swordtails spend significantly greater time associating with males, regardless of size, as compared with female mosquitofish. Female mosquitofish spend significantly less time in the association zone when males are present as compared to when females are present or no fish at all. This result suggests some level of male avoidance by mosquitofish females, a pattern not observed in swordtail females (Fig. 2a).

Although many studies show no evidence of female mate preference within a coercive mating system, a few studies, including this study, reveal that some mosquitofish species do exhibit a bias toward particular male characteristics (Bisazza & Pilastro 2000; Gould *et al*. 1999; Kahn *et al*. 2010). We find that these biases tend to be weaker than those of female swordtails. More specifically, the proportion of time spent with large males as compared to small males is marginally different between these species and when we increase the sample by including the behavior of female swordtails from Cummings *et al*. (2008), the results reveal a significant interaction in the proportion of time spent with large males as compared to small males between these species. A general avoidance of males coupled with a bias for the larger male (in a large/small male context) may reflect attempts to minimize harassment as large male mosquitofish guard females from harassment (Bisazza & Marin 1995). Because male harassment has a large impact on female foraging efficiency (Pilastro *et al*. 2003), it is potentially less costly for females to associate with larger males. Thus, in

Table 1: Expression of *neuroserpin*, *neuroligin-3* and *NMDA-R* and behavior across treatments (male-exposed, female-exposed and asocial) These three genes were examined in relation to association bias and total transits

	r/*P*		
	Neuroserpin	Neuroligin-3	NMDA-R
Association bias			
Gambusia affinis			
♂♂	−0.62/0.054	−0.63/0.049	−0.18/0.67
♀♀	−0.289/0.42	−0.310/0.38	−0.283/0.43
Asocial*	−0.254/0.54	−0.266/0.53	−0.313/0.45
Xiphophorus nigrensis			
♂♂	0.64/0.01	0.52/0.058	−0.72/0.008
♀♀	−0.156/0.74	−0.111/0.81	0.054/0.91
Total transits			
Gambusia affinis			
♂♂	0.186/0.60	0.241/0.50	0.011/0.97
♀♀	−0.221/0.54	−0.246/0.49	−0.220/0.54
Asocial	0.731/0.04†	0.729/0.04†	0.654/0.07
Xiphophorus nigrensis			
♂♂	−0.172/0.55	0.070/0.81	0.034/0.91
♀♀	0.200/0.66	0.133/0.77	0.036/0.93
Asocial‡	−0.166/0.96	−0.164/0.91	—

Total transits are defined as the number of times the test female crossed the boundary between the association zone and the neutral zone. Transits provide an estimate of exploratory activity.
*Activity measures in the asocial condition were examined in relation to bias for left or right side rather than association with another fish.
†Values significant at the 0.05 alpha level do not meet FDR requirements.
‡Behavior was not recorded for swordtail in the asocial condition in this study. We present total transits from female swordtails tested for Cummings *et al*. 2008 as a point of reference.

the mosquitofish mating system sexual conflict is heightened resulting in substantially different social behavior from that of the female swordtails, particularly when males are present.

Comparing gene expression in the brains of related species with divergent behavioral phenotypes is valuable for understanding the underlying mechanisms of behavior (de Belle *et al*. 1989; Ben-Shahar 2005; Ben-Shahar *et al*. 2002; Haesler *et al*. 2004; Hammock & Young 2004). Here, the differences in gene expression patterns between the two species are salient. Male-exposed mosquitofish exhibit negative relationships with association bias across two of three preference-associated genes, whereas male-exposed female swordtails exhibit positive relationships between association bias and gene expression for the same two genes (Fig. 3a–f), while both species display no pattern with a control gene. These results are consistent with previous whole brain expression patterns for NS and NLG-3 in female swordtails ($r = 0.76$, $P < 0.001$; $r = 0.77$, $P < 0.001$ respectively; Cummings *et al*. 2008). Further, there is also a significant interaction between gene expression–behavior relationships and species in male-exposed females for NS and NLG-3 that is largely driven by the fact that patterns of NS and NLG-3 whole brain gene expression are directly opposing one another in male-exposed females. In contrast, female-exposed fish show no significant relationships between whole brain gene expression and association bias in mosquitofish or swordtails (Table 1).

Mate preference includes an information-gathering phase and therefore, an exploratory component. It is possible that these genes may serve as part of the molecular substrates that gave rise to exploratory behavior during a mate choice context, and are therefore upregulated in swordtails while males are present. Interestingly, there is a significant relationship between NS and NLG-3 expression and total activity in female mosquitofish in the asocial condition (Table 1), whereas earlier work on the swordtails did not uncover any significant relationships between gene expression and activity under asocial conditions (Cummings *et al*. 2008) or with total transits (see Table 1). Thus, it is possible that these genes underlie exploratory behavior in a context-dependent manner. For instance, these genes may facilitate synaptic-plasticity during exploration but are inhibited in female mosquitofish when males are present. In this respect, gene induction would mirror female behavior because social approach behavior is also inhibited when males are present. On the contrary, in a species that displays robust female mate choice such as the swordtail, females exhibit strong association bias and clear social approach behavior when males are present. In this system, gene induction also mirrors the female swordtail's behavior, such that the positive relationship with approach behavior is seen only in the male-exposure condition.

The opposing NS and NLG-3 expression–association bias relationship between these species with diverging preference behavior is especially interesting considering the function of these genes in regulating social behavior in other taxa. NS deficits and overexpression in mice results in behavior characterized by reduced locomotion in novel environments or other anxiety-producing situations as well as neophobia to novel objects (Madani *et al*. 2003). Irregularities in the function of NLGs are presumed to lead to a loss of synaptic function associated with social behavior deficiencies, such as autism (Radyushkin *et al*. 2009; Südhof 2008). NMDA-R is prominently involved in the neural substrate of event-related LTP in the amygdala (Lynch 2004), which is involved in regulating behavioral responses, including social-defeat changes in behavior (Day *et al*. 2011). Thus, each of these genes facilitates the cognitive and emotional processes regulating a variety of social behaviors.

Individually, NS, NLG-3 and NMDA-R represent excellent candidate genes underlying mate preference behavior whereas together, they represent a putative molecular pathway involved in mate preference behavior. These genes share a function in regulating neural and synaptic plasticity, which is interesting with respect to mate choice because this behavior is a complex process that requires information-gathering and decision-making phases, processes that are important to a suite of exploratory behaviors, not simply mate choice. It is possible that these genes underlie exploration-related neural plasticity and may exhibit social context-dependent expression. For instance, choosy females must acquire information about multiple males before making mate decisions and those females may employ a variety of

strategies, such as choosing the best male in a fixed sample (i.e. the best of *n* approach) during a mate searching task (Real 1990). Such mate searching strategies may require animals to possess mechanisms facilitating the rapid acquisition of new information about the world around them (including social information), a process that occurs during associative or spatial learning and is a neural plasticity-dependent event. Therefore, in animals displaying robust mate preference, exploratory behavior in a social context in which males are present may be associated with an upregulation of plasticity-related genes such as these. On the contrary, in an animal with heightened sexual conflict (and therefore, minimal female mate preference), the presence of males may inhibit such gene expression, whereas exploratory behavior in an asocial environment may be associated with the upregulation of these genes. We see this relationship for two of the three genes examined, NLG-3 and NS. In contrast, our data reveal a negative relationship between transcriptional regulation of NMDA-R and association bias in female swordtails. It is possible that the relationship between behavior and NMDA-R must be examined at the protein level or through changes in the kinetics of NMDA-R. Nonetheless, NLGs, NS and NMDA-R are all genes that regulate the molecular machinery underlying synaptic plasticity-related events such as learning (Biswas *et al*. 2010; Lynch 2004; Miranda & Lomas 2006) and thus, may serve as components of molecular substrates by which mate choice behavior evolves.

Taken as a whole, our results reveal that two related poeciliids species, the Western mosquitofish and the Northern swordtail, exhibit contrasting behaviors and whole brain gene expression patterns with respect to female mate choice. Although our experimental conditions attempted to isolate differences between mating systems, we cannot rule out that species-level differences in developmental, ecological and evolutionary processes may be reflected in the species-specific patterns of gene expression. Nonetheless, gene–behavior relationships reveal that NS and NLG-3 exhibit dramatic differences in expression profiles between these two phylogenetically similar species with contrasting mating systems. Ongoing work reveals that NS and NLG-3 are significantly upregulated specifically in brain regions commonly associated with learning and social behavior in male-exposed swordtail females. This differential expression occurs in females exhibiting strong mate preference response as compared to females exhibiting weak preference response to male exposure (Wong 2011). These results, in addition to the results presented here, indicate that these genes may be prime modulators of mate preference behavior. Moreover, the functional relationship between these genes allows us to formulate and test hypotheses concerning the role of synaptic plasticity and learning as part of the neural substrates by which mate search strategy and even mate preference behavior evolves.

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Acknowledgments

The authors thank undergraduate students Jason Sumpter and Michael Pham for their assistance in the lab as well as graduate students Ryan Wong, Eben Gering and Chad Brock for technical assistance and advice. This work was supported by NSF IOS 0843000 to M.E.C. We would also like to thank the Mexican government for collecting permits (DGOPA07311-13709-2261) and the Brackenridge Field lab for animal care facilities.

Supporting Information

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

Table S1: (a) Cloning gene parameters: Degenerate primers were designed using CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) and were used to clone the following genes. Consensus sequences were derived from known fish protein sequences (*X. nigrensis, R. danio, A. burtoni*). The reaction parameters included a denaturing cycle (94°C for 2 min) followed by 30 amplification cycles (94°C denaturing for 30 s, 55°C annealing for 1:30 min and 72°C elongation for 1:30 min) followed by a final 10-min cycle for elongation (72°C). (b) qPCR parameters: 2 min at 50°C, 10 min at 95°C for denaturing, followed by 40 cycles at 15 s at 95°C, 30 s at 60°C and 30 s at 72°C.

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