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Plasticity of the mate choice mind: courtship evokes choice-like brain responses in females from a coercive mating system

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Female mate choice is fundamental to sexual selection, and determining molecular underpinnings of female preference variation is important for understanding mating character evolution. Previously it was shown that whole-brain expression of a synaptic plasticity marker, *neuroserpin*, positively correlates with mating bias in the female choice poeciliid, *Xiphophorus nigrensis*, when exposed to conspecific courting males, whereas this relationship is reversed in *Gambusia affinis*, a mate coercive poeciliid with no courting males. Here we explore whether species-level differences in female behavioral and brain molecular responses represent 'canalized' or 'plastic' traits. We expose female *G. affinis* to conspecific males and females, as well as coercive and courting male *Poecilia latipinna*, for preference assays followed by whole-brain gene expression analyses of *neuroserpin*, *egr-1* and *early B*. We find positive correlations between gene expression and female preference strength during exposure to courting heterospecific males, but a reversed pattern following exposure to coercive heterospecific males. This suggests that the neuromolecular processes associated with female preference behavior are plastic and responsive to different male phenotypes (courting or coercive) rather than a canalized response linked to mating system. Further, we propose that female behavioral plasticity may involve learning because female association patterns shifted with experience. Compared to younger females, we found larger, more experienced females spend less time near coercive males but associate more with males in the presence of courters. We thus suggest a conserved learning-based neuromolecular process underlying the diversity of female mate preference across the mate choice and coercion-driven mating systems.

Keywords: *early B*, *egr-1*, female preference, *Gambusia affinis*, *neuroserpin*, phenotypic plasticity, *Poecilia latipinna*, learning, synaptic plasticity, *Xiphophorus nigrensis*

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Female mate choice is an important aspect of sexual selection and mating system diversity (Darwin 1859; Guilford & Dawkins 1991). In mating systems where coercive male harassment is the predominant mating tactic, female mate bias is usually muted relative to systems with courting males, where female choice directly determines male mating success. Female responses toward males in these systems differ, yet little is known as to whether canalized (invariant with mating system) or plastic (variation due to environmental stimuli) mechanisms underlie these differences. Here we ask if female 'choice-typical' behavioral and neural responses to males are constrained to 'choice' species or can we recover it in a 'coercive' species with courting male stimuli?

We investigate this question with Poeciliidae, a family of livebearer fishes exhibiting a spectrum of mating systems from female choice to male coercion (Bisazza 1993; Farr 1989). For instance, while male mating strategy is primarily coercive in *Gambusia affinis* in which females show little mate preference (Farr 1989), the mating systems of *Xiphophorus nigrensis* and *Poecilia latipinna* possess both coercer and courter males and distinctive female preference for large courting males (Simanek 1978; Luckner 1979; Woodhead 1985; Ryan & Causey 1989).

Research to date has already established that whole-brain expression levels of two genes associated with synaptic plasticity, *neuroserpin* and the immediate early gene *egr-1* (Lee *et al.* 2012; Lee *et al.* 2012; Miranda & Lomas 2006) are positively correlated with female bias toward a favored male stimulus in female *X. nigrensis* (Cummings *et al.* 2008). In contrast, we find a negative covariance between these same genes and behavior in *G. affinis*, where females exhibit a negative correlation between gene expression and strength of preference in the presence of coercive conspecific males (Lynch *et al.* 2012). These relationships scale with individual variation in female strength of preference toward different male phenotypes and suggest that context-specific covariance between female neuromolecular and behavior responses may not be evident in group-level comparisons. Further, our results suggest the intriguing possibility that the same synaptic plasticity-associated gene module might mediate female preference in both species, but be differentially modulated by male phenotype in choice or coercive mating systems. Unlike *X. nigrensis* in which both courter and coercer males exist, all *G. affinis* males are coercers (Bisazza & Marin 1991; Farr 1989), therefore the differential brain gene expression in *G. affinis* may be due to the absence of courter males, rather than distinct species-specific differences in female brain response.

If gene expression patterns associated with female preference are conserved but extrinsically influenced by various male stimuli, then exposing *G. affinis* females (from a coercion-driven mating system) to courting male phenotypes should result in choice-like gene responses. Because *G. affinis* females showed preference for novel male models with enlargement of dorsal fins over their conspecific male models (Gould *et al.* 1999), we hypothesized that male sailfin molly (*P. latipinna*), known for enlarged dorsal fins, would be salient stimuli for female *G. affinis*. Here, we conduct preference tests with *G. affinis* females exposed to different combinations of coercive and courting phenotypes using conspecific and heterospecific *P. latipinna* males, and examine female behavior and brain gene expression patterns. In addition, we investigate age effects on female mating bias across these choice conditions.

Material and methods

Behavior

The behavioral tests were conducted during October 2011 to February 2012. Female and male *G. affinis* and male *P. latipinna* fish were collected as adults from a pond on the campus of University of Texas at Austin. The standard length (SL) of the focal *G. affinis* female fish ranged from 28.6 to 45.9 mm. All fish were collected from the same pond and were assumed to be sexually experienced and familiar with the heterospecific species. To control for laboratory experience, all testing subjects were held in the same fish room under identical lighting/food procotols. To ensure motivation to associate with stimulus individuals, each fish was isolated for at least 2 weeks prior to behavioral observations as in Cummings *et al.* (2008) (Lynch *et al.* 2012; Ramsey *et al.* 2012; Wong *et al.* 2012). The SL (mean ± standard deviation) of stimulus fish were: 37.68 ± 3.09 mm for conspecific large females (C_F), 31.40 ± 3.40 mm for conspecific small females (C_f), 26.28 ± 2.33 mm for conspecific large males (C_M), 21.06 ± 1.36 mm for conspecific small males (C_m), 33.96 ± 1.28 mm for heterospecific coercer males (*P. latipinna*, H_{Cr}) and 53.95 ± 0.65 mm for heterospecific courter males (*P. latipinna*, H_{Ct}). As in Luckner (1979) and Simanek (1978), we used the following criteria to distinguish *P. latipinna* male phenotypic class: after ensuring that all the males were mature with complete gonopodium, courting males (H_{Ct}) had distinctive orange on dorsal fins, dorsal fins long enough to reach the base of the caudal fins, and iridescent blue and orange coloration on caudal fins while coercive males (H_{Cr}) lacked any such coloration and had short dorsal fins. Note that courting *P. latipinna* males tend to be larger than the more moderately sized coercive *P. latipinna* males; yet both are significantly larger than *G. affinis* males. *G. affinis* females were randomly assigned to one of the five behavior treatments: asocial (empty stimulation zones, $n = 7$), small vs. large female conspecifics (C_fC_F, $n = 7$), small vs. large male conspecifics (C_mC_M, $n = 14$), small conspecific vs. coercive heterospecific males (C_mH_{Cr}, $n = 15$), and small conspecific vs. courting heterospecific males (C_mH_{Ct}, $n = 7$).

Behavior tests followed procedures detailed by Cummings *et al.* (2008). The two stimuli were placed on either sides of the experimental tank. The focal female fish were isolated from the stimuli by Plexiglas dividers. The center compartment of the tank was subdivided into three zones: the central neutral zone and two association zones on the sides. The focal female mosquitofish was placed inside an opaque tube at the center of the neutral zone during a 5 min acclimatization period, and then released into the center compartment. This was followed by two 15-min observation periods in which the following behavior variables were recorded: time focal females spent in either of the association zone and transits that the females make from the association zones to the neutral zone. At the end of the first 15 min, the two stimuli were switched to eliminate the effect of side bias of the focal fish. This setup restricts female assessment of male stimuli to visual mode only. Total transits (movements out of the association zones) along with time spent in each association zone were recorded for each focal female. Association bias, an index for preference strength, was calculated as the proportion of association time spent in the association zone of individual *a*, where time with individual *a* > time with individual *b* (Cummings *et al.* 2008); and total association time (time spent in both association zones) was calculated as a measure of female motivation to interact with social stimuli.

Gene expression

We tested *neuroserpin*, a neuroplasticity gene known to exhibit contrasting expression patterns in coercive vs. choice females as well as *egr1*, previously associated with mate choice (Cummings *et al.* 2008). As a control, we also included *early B*, previously shown to not be associated with mate choice exposure in either *X. nigrensis* or *G. affinis* females exposed to conspecific males (Cummings *et al.* 2008; Lynch *et al.* 2012). We examined the expression level of *neuroserpin*, *egr-1* and *early B* in the brains of the focal females after the behavior treatments. Immediately after the end of each behavior trial, the focal females were sacrificed and brain tissues were collected and stored in RNA later (Applied Biosystems, Carlsbad, CA, USA). Each individual brain was immersed in RNAlater solution in a 2 ml tube at 4°C with gentle shaking for 24 h before the RNA later solution was taken out and brain tissue tubes immediately transferred to −80°C storage until RNA extraction. All experimental procedures were approved by IACUC at the University of Texas at Austin (protocol number: AUP-2010-00148). RNA extraction of the brain samples was conducted with Trizol (Invitrogen, Carlsbad, CA, USA). The extracted RNA was DNase-treated with turbo DNA-free kit (Applied Biosystems) prior to cDNA synthesis with Superscript First-Strand Synthesis (Invitrogen). The cDNA samples were cleaned with Amicon Ultra Centrifugal Filter units (Merck Millipore, Billerica, CA, USA). Gene sequencing, cloning and primer pair design were conducted by Lynch *et al.* (2012). Gene expression was quantified by qPCR with SYBR green detection on ABI prism 7900 qPCR machine (Applied Biosystems) in which each sample was run in triplicate. The qPCR result was initially analyzed with Applied Biosystems Real-Time PCR system software (ViiA™ 7). The gene expression levels were normalized by cDNA input quantities measured by RiboGreen RNA quantification assay with Quant-iT RiboGreen RNA reagent (Molecular Probes, Invitrogen), as in (Cummings *et al.* 2008; Lynch *et al.* 2012; Ramsey *et al.* 2012). Ribogreen reagent binds RNA and single-stranded cDNA with equal efficiency (see Cummings *et al.* 2008 Supplemental Table 2 for validation), therefore we normalized our raw qPCR values with input template concentration using cDNA – the same cDNA preparation used in the qPCR assays. This allows us to avoid potential variation in template quantity introduced during handling steps in producing qPCR quality cDNA from RNA. The qPCR standard curve was derived from a sample of pooled *G. affinis* brains ($n = 5$) scaled up but otherwise processed the same as individual sample preparations. The standard curves for all qPCR assays were prepared from this pooled sample. Standard curves were serially diluted (ranging from 98 ng/μl to 4.9 pg/μl concentrations) such that the target gene abundance for each sample fell on the curve. Thus the target gene qPCR output for each of the sample can be inferred relative to the standard curve on the same reaction plate. To determine the normalized expression value of each target gene controlling for the input cDNA concentration of the samples, we derived the residuals from a linear regression of target gene qPCR output onto the input cDNA concentration as determined from the RiboGreen assay (*see Normalized gene expression level below*).

Statistical analysis

Behavior across groups

Behavior measures were not normally distributed, so Kruskal–Wallis one-way analysis of variance was performed to compare the difference of association bias, total association time, and total transits across the male-exposure conditions (C_mC_M, C_mH_{Cr} and C_mH_{Ct}). If the *p*-value was less than 0.05, pairwise Wilcoxon Mann–Whitney tests with Benjamini–Hochberg FDR corrections (Benjamini & Hochberg 1995) were performed. Wilcoxon Signed Rank test were used to examine within-treatment preference.

Normalized gene expression level

To normalize gene expression levels to input cDNA, linear regression models were constructed for each gene. The response variable was qPCR value (raw qPCR quantity estimates based on standard curve estimates and averaged across the triplicates) and the predictor variable was the initial sample cDNA quantity (measured by Ribogreen). The residuals derived from the regression models represent the deviation of the observed gene cDNA quantity from the predicted gene cDNA quantity based on the brain sample cDNA concentrations, and thus can be used to infer normalized gene expression level as used by Lynch *et al.* (2012) and Ramsey *et al.* (2012).

To ensure that the distributions of the variables agree with the assumption of the linear regression model, we power-transformed the variables that did not pass Shapiro–Wilk normality tests. For each of the variables that failed Shapiro–Wilk normality test, the power transformation coefficient was estimated by the power transformation function of the Car package in R (Box & Cox 1964). The predictor variable (ribogreen measures of input cDNA) passed the normality test ($p > 0.05$), but the response variable (qPCR output quantity for each gene) did not ($p < 0.05$). The distribution of the qPCR quantities of the three genes exhibited right-skewness and the power transformation estimates of the *neuroserpin*, *egr-1* and *early B* qPCR quantities were respectively: 0.22, 0.25 and 0.29. After transforming the qPCR raw quantities of the three genes with a power of 0.22, both *neuroserpin* and *egr-1* qPCR quantities passed Shapiro–Wilk normality test ($p > 0.05$), but not for *early B* qPCR quantities ($p = 0.013$). Because the *egr-1* and *early B* residuals derived from 0.25 and 0.29 transformation showed similar patterns on all the subsequent analyses, we transformed qPCR quantities of the three genes with 0.22 so that further analyses of the three genes were comparable. The regression model of each gene was constructed as following:

$$y^{0.22} = \beta_0 + \beta_1 x + \epsilon,$$

where y represents the qPCR output quantities of each gene (*neuroserpin*, *egr-1* and *early B*) respectively, x is the brain sample input cDNA quantity, β_0 is the intercept, β_1 is the predictor coefficient and ϵ is the residual. The regression models were intrinsically linear and the residuals from these models preserved the relative levels of normalized gene expression.

Association of gene and behavior

With the residuals of the three genes, we compared gene expression levels across different treatment groups with Kruskal–Wallis one-way analyses of variance (ANOVA) because of the small sample size per group. Post hoc pairwise Wilcoxon Mann–Whitney tests followed by FDR correction were performed if the p value of Kruskal–Wallis ANOVA was <0.05.

Previous studies of mate choice molecular mechanisms revealed species-level differences in the sign of the covariance between female neuromolecular response and behavioral response (Lynch *et al.* 2012). Group-level comparisons of gene and behavior expression separately will not always reveal underlying gene by behavior covariance structure within groups, particularly when comparing groups with different behavioral tendencies. Thus, in addition to analyzing gene expression and behavior differences at the group level, we also examine gene by behavior covariance relationships within each treatment group. Specifically, we examined the relationship between gene expression level and association bias across each social treatment group (C_fC_F, C_mC_M, C_mH_{Cr} and C_mH_{Ct}) by generating linear regression models each with a response variable (gene residual) and predictor variables (association bias and social treatment category). We developed two levels of analysis to evaluate the gene-by-behavior covariance structure: Model 1 (included all social treatment groups) and Model 2 (included only the heterospecific male exposure social groups to explore male type-dependent gene-by-behavior associations). We also tested the difference of regression slopes across the treatment groups. Because we used the same predictors for each gene, we conducted Benjamini–Hochberg FDR correction (Benjamini & Hochberg 1995) for the p-values of the ANOVA of each multiple linear regression model.

Association of age and behavior

To examine the effect of female standard length (SL, a proxy of age) on association behavior, we built linear regression models with response variables (association bias, total association time, or total transit) and predictor variables (SL and social treatment group). We again analyzed with regression models at two levels: Model 1 (included all four social treatment groups) and Model 3 (comparing courting male group (C_mH_{Ct}) to coercive male groups ($C_mC_M + C_mH_{Cr}$)). As above, FDR correction was performed for each multiple linear regression model.

Results

Context-specific association bias

Within the social treatment groups, focal females exhibited significant differences in association time with specific stimuli only under C_mH_{Cr} conditions (Table 1). However, when comparing across male-exposed social groups, we found no significant differences in focal female total association time, total transits or association bias (Table 2; Fig. 1a).

Context-specific gene expression

Looking across groups, the expression levels of *neuroserpin*, *egr-1* and *early B* were not significantly different, although there were consistent non-significant trends of reduced transcription level in C_mH_{Cr} than in C_mH_{Ct} among male-exposure treatments (Fig. 1b–d; Kruskal–Wallis one-way ANOVA, *neuroserpin*: $p = 0.08$; *egr-1*: $p = 0.09$; *early B*: $p = 0.10$).

Context-specific correlations between gene expression and behavior

Modeling the relationship between gene expression and association bias revealed context-specific associations that were highly significant for females exposed to heterospecific courting males but only moderately predictive when all social groups were included. Regression ANOVAs for Model 1 analyses (all social treatments included; C_fC_F, C_mC_M, C_mH_{Cr} and C_mH_{Ct}) indicated overall significance for *neuroserpin* and *egr-1* only (Table 3). While the overall model for *early B* was not significant, association bias appeared to be a significant factor in predicting gene response. The interaction between association bias with social treatment conditions significantly explains the residuals of all three genes (Table 3). Regression models predicting the residuals of *neuroserpin*, *egr-1* and *early B* by association bias across all social treatments

Table 1: Association time (s, mean ± SD) of focal *Gambusia* females in each side of the association zone (with large or small stimulus) by treatment group

	Association time (s, mean ± SD)		Wilcoxon signed rank test	
Group	Small stimulus	Large stimulus	*T*	*P*-value
C_fC_F	758 ± 310	771 ± 360	12	0.81
C_mC_M	713 ± 280	812 ± 309	66	0.43
C_mH_{Cr}	462 ± 372	1055 ± 422	104	0.01
C_mH_{Ct}	637 ± 242	722 ± 161	19	0.47

Table 2: Comparison across male-exposure groups (C_mC_M, C_mH_{Cr}, C_mH_{Ct}) of focal female association bias, total association time (s) and total transits (mean ± SD values)

	Mean ± SD			Kruskal–Wallis one-way ANOVA		
Behaviors/group	C_mC_M	C_mH_{Cr}	C_mH_{Ct}	X^2	d*f*	*P*-value
Association bias	0.63 ± 0.11	0.75 ± 0.16	0.61 ± 0.06	5.17	2	0.08
Total association time	1525.43 ± 211.57	1517.13 ± 248.80	1359.14 ± 264.56	2.50	2	0.29
Total transits	25.43 ± 12.67	25.33 ± 33.24	29.57 ± 17.92	2.74	2	0.25

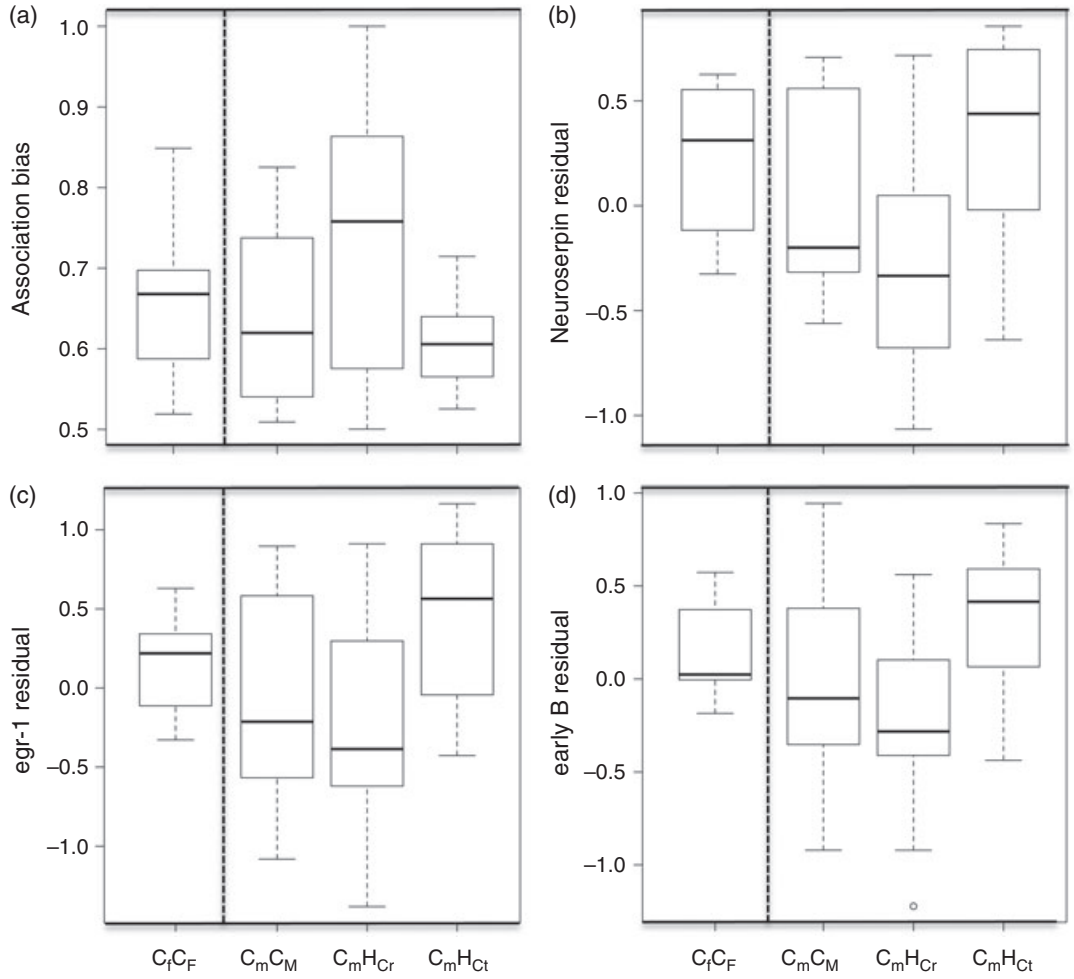

Figure 1: Behavior and gene expression by social exposure group. Boxplot of association bias (a) and *neuroserpin* (b), *egr-1* (c) and *early B* (d) residuals across large vs. small female conspecifics group (C_fC_F) and male-exposure conditions: small vs. large male conspecifics (C_mC_M), small conspecific male vs. coercive heterospecific male (C_mH_{Cr}) and small conspecific male vs. large courting heterospecific male (C_mH_{Ct}).

(C_fC_F, C_mC_M, C_mH_{Cr} and C_mH_{Ct}) suggested that the linear relationships are significant only when females are exposed to courting heterospecific males, C_mH_{Ct} (Table 4, Fig. 2a,c,e).

Examining the relationship between gene expression and association bias across all male-exposed social contexts (C_mC_M, C_mH_{Cr} and C_mH_{Ct}) revealed significance for *early B* only (*neuroserpin*: $p = 0.15$; *egr1*: $p = 0.27$, *early B*: $p = 0.04$). However, when we confine the analysis to only the heterospecific male groups (C_mH_{Cr}, and C_mH_{Ct}, Model 2) we found overall significance for all the three genes (Table 3). The residuals of *neuroserpin* and *early B* are significantly explained by association bias (Table 3), and the interaction between association bias and heterospecific male social group is a significant factor for all three genes (Table 3,

Table 3: The regression models that predict gene (*neuroserpin*, *egr-1* and *early B*) residuals with AB (association bias) and social group treatments (Model 1: all social groups: C_fC_F, C_mC_M, C_mH_{Cr} and C_mH_{Ct}; and Model 2: heterospecific male groups only: C_mH_{Cr} and C_mH_{Ct}). Analyses of variance of models that predict gene residuals by AB, across all social treatments (Model 1), and in heterospecific male groups (Model 2)

Model 1					
Response (gene residual)	Factors				
	Groups, $F(3, 35)$ (P)	Association bias, $F(1, 35)$ (P)	Groups × association bias, $F(3, 35)$ (P)	$F(7, 25)$	P-value
neuroserpin	2.66 (0.06)	2.46 (0.13)	4.28 (**0.01**)	3.33	**0.008**
egr-1	1.93 (0.14)	1.62 (0.21)	3.45 (**0.03**)	2.54	**0.03**
early B	1.83 (0.16)	4.65 (0.04)	3.35 (**0.03**)	2.88	0.17
Model 2					
Response (gene residual)	Factors				
	Groups, $F(1, 18)$ (P)	Association bias $F(1, 18)$ (P)	Groups × association bias, $F(1, 18)$ (P)	$F(3, 18)$	P-value
neuroserpin	3.77 (0.07)	6.13 (**0.02**)	9.11 (**0.007**)	6.34	**0.004**
egr-1	4.35 (0.07)	3.66 (0.07)	8.18 (**0.01**)	5.4	**0.008**
early B	3.63 (0.07)	8.20 (**0.01**)	10.48 (**0.005**)	7.44	**0.002**

The bolded P-values indicate significance that survived FDR correction.

Table 4: Regression coefficients (reflect the intercept or slope of the linear relationship between AB and gene residuals) and P-values for each group-specific predictor, along with the overall R^2 and R^2-adjusted for Model 1

Model 1							
Response (gene residual)		Estimate (P-value)					
		C_fC_F	C_mC_M	C_mH_{Cr}	C_mH_{Ct}	R^2	R^2_{adj}
neuroserpin	Intercept	0.47 (0.67)	−1.49 (0.26)	0.41 (0.74)	−4.52 (0.03)		
	AB coefficient (slope)	−0.40 (0.81)	2.05 (0.30)	−1.15 (0.53)	7.54 (**0.03**)	0.4	0.28
egr-1	Intercept	0.67 (0.63)	−1.70 (0.31)	0.29 (0.85)	−5.61 (0.04)		
	AB coefficient (slope)	−0.81 (0.70)	2.38 (0.34)	−0.81 (0.72)	9.64 (0.03)	0.34	0.2
early B	intercept	0.26 (0.80)	−0.42 (0.74)	0.73 (0.54)	−3.98 (0.05)		
	AB coefficient (slope)	−0.15 (0.92)	0.41 (0.83)	−1.48 (0.39)	6.77 (**0.04**)	0.37	0.24

The bolded P-values indicate significance that survived FDR correction.

Fig. 2b,d,f). In the presence of courting male heterospecifics (C_mH_{Ct}), association bias linearly predicted the residuals of *neuroserpin*, *egr-1* and *early B* (Table 5, Fig. 2b,d,f). Meanwhile, in the presence of coercive male heterospecifics (C_mH_{Cr}), association bias only predicts the expression of *neuroserpin* and *early B* (Table 5, Fig. 2b,f).

Stimulus-dependent size (age) effect on association behaviors

To test the role of age/experience on the plasticity of mate preference behavior, we examined the relationship between standard length (SL, a proxy of age) and behavior (association bias, total association time and total transits) across all social treatments (Model 1). Standard length does not significantly predict association bias ($p = 0.35$) nor total association time (although there was a trend, $p = 0.06$; Fig. 3a) displayed by focal females across all social groups. However, SL significantly predicts total transits exhibited by focal females (Table 6, Fig. 3c). SL is the significant predictor for total transits across heterospecific male groups, C_mH_{Cr} and C_mH_{Ct} (Table 7, Fig. 3c), but it significantly predicts total association time only within the courting heterospecific group, C_mH_{Ct} (Table 7, Fig. 3a). The interaction between SL and social group significantly explained total transits and total association time (Table 6).

We then examined the effect of female age and/or experience on total association time and total transits across male exposure groups (C_mH_{Ct}, C_mC_M and C_mH_{Cr}, Model 3). ANOVAs for each response variable were significant (Table 6), and revealed that both total transits (Fig. 3d) and total association time (Fig. 3b) are significantly predicted by the interaction between SL and male exposure (Table 6, Fig. 3). Regression models show that SL is the significant predictor for total association time and total transits both within coercive (C_mC_M and C_mH_{Cr}) and courting (C_mH_{Ct}) male exposure groups, but in different directions (Table 8, Fig. 3b,d).

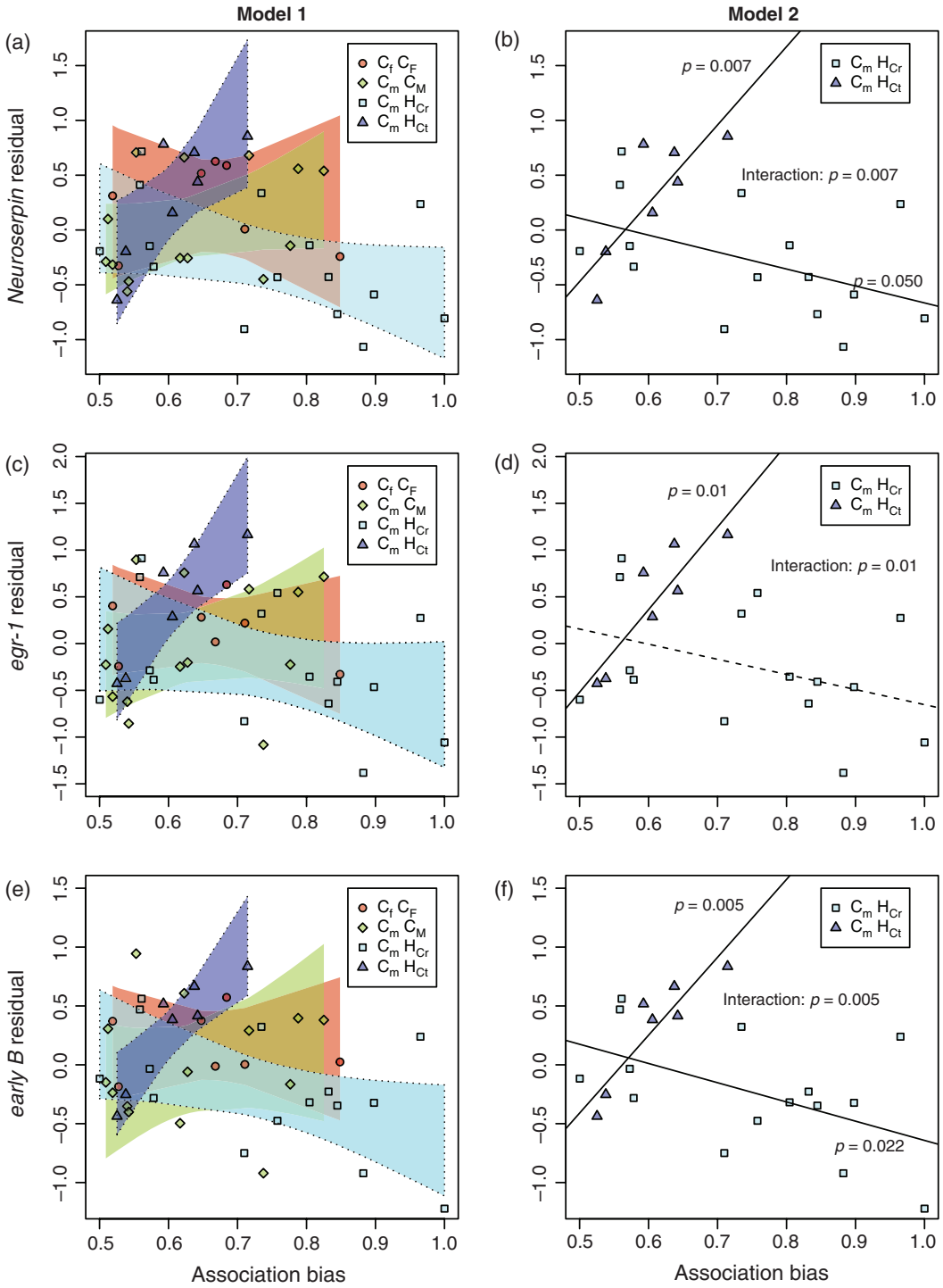

Figure 2: Divergence in female gene-by-preference behavior covariance is driven by exposure to different male phenotypes (coercers vs. courters). Scatter plot with 95% confidence regions of generalized linear models of gene residuals (a) *neuroserpin*, (c) *egr-1*, (e) *early B* and association bias across each social treatment group: C_fC_F (red), C_mC_M (green), C_mH_{Cr} (light blue), C_mH_{Ct} (dark blue). Scatter plot of gene residuals (b) *neuroserpin*, (d) *egr-1*, (f) *early B* and association bias of focal female fish in the two heterospecific male exposure groups: C_mH_{Cr} (coercers, light blue squares) and C_mH_{Ct} (courters, dark blue triangles). Significant relationships are outlined by dashes in (a,c,e) and by solid regression lines in (b,d,f). See Tables 3–5 for statistical results.

Table 5: Regression coefficients (reflect the intercept or slope of the linear relationship between AB and gene residuals) and *P*-values for each group-specific predictor, along with the overall R^2 and R^2-adjusted for Model 2

		Model 2			
Response (gene residual)		Estimate (*P*-value)			
		C_mH_{Cr}	C_mH_{Ct}	R^2	R^2_{adj}
neuroserpin	Intercept	0.88 (0.13)	−4.92 (**0.01**)		
	AB coefficient (slope)	−1.55 (0.05)	8.69 (**0.007**)	0.51	0.43
egr-1	Intercept	0.96 (0.19)	−1.62 (**0.02**)		
	AB coefficient (slope)	−1.62 (0.10)	10.45 (**0.01**)	0.47	0.39
early B	Intercept	0.99 (0.06)	−4.71 (**0.008**)		
	AB coefficient (slope)	−1.64 (0.02)	8.25 (**0.005**)	0.55	0.48

The bolded *P*-values indicate significance that survived FDR correction.

Discussion

Females avoid males in coercive mating systems, and this avoidance may be mediated in the brain via canalized neuromolecular responses that are invariant across variation in male phenotypes, or may be plastic and responsive to differences in male behavior. Here we find that differential mate choice contexts elicited plastic responses in female preference behaviors, and importantly, significant differences in the brains of females exposed to different male tactics. *Gambusia* females exhibited context-dependent relationships between behaviors and gene patterns, suggesting female plasticity of the behavior-gene response. By introducing heterospecific coercive and courting male phenotypes to female *Gambusia* we observed (1) negative correlations between gene expression of all three genes examined and bias behavior with *coercive* males, as well as (2) positive correlations between these same genes and bias behavior with *courting* males (Fig. 2, Tables 3–5). This within-species plasticity mirrors that of the species-level differences we have previously observed between mate choice and coercive taxa (Lynch *et al.* 2012) and suggests that it is the mating phenotype of males that drives differential female response rather than canalized neuromolecular processes intrinsic to species or mating system. These results suggest a potential conserved brain module that is plastic in nature governing female response to courting males; and that differential modulation of common neural substrates produce distinct female responses in divergent mating systems.

In this study, we reversed 'coercive-like' and recovered 'mate choice-like' neuromolecular patterns in females of a coercive mating system by introducing a heterospecific courting male. The expression patterns of synaptic plasticity markers, *neuroserpin* and *egr-1*, in the brains of *G. affinis* females in the presence of courting heterospecific males in our study (Fig. 2b,d) mimic the previously described pattern of *X. nigrensis* females from a mate choice system with courting phenotypes (Cummings *et al.* 2008; Lynch *et al.* 2012), indicating that the engagement of synaptic plasticity-associated markers underlying female preference might be related to the presence of an ornamented courting male. This study suggests that the presence of specific male phenotypes can potentially determine whether gene modules associated with synaptic plasticity are inhibited or expressed. Previous research suggested that females in mate choice taxa (*X. nigrensis*) are activating synaptic plasticity modules in the brain when exposed to ornamented, courting male phenotypes (Cummings *et al.* 2008; Lynch *et al.* 2012; Wong *et al.* 2012), potentially due to synaptic connection modulation required for mate assessment. This study provides a unique body of evidence that suggests differential dynamic gene responses in female brains are invoked by distinct male phenotypes. Future work should determine whether the localized expression patterns of these genes are in the same forebrain regions associated with reward and social-decision making processes as those of *X. nigrensis* [Dm and Dl, dorsomedial telencephali and dorsolateral telencephali (Wong *et al.* 2012)], as well as directly test the role of social experience and plasticity in female brain and behavioral responses to differing male phenotypes.

Early B has not been previously associated with female association bias toward conspecific males in either species, although it has been linked to conspecific female exposure in *X. nigrensis* (Cummings *et al.* 2008). As in Lynch *et al.* (2012), we found no relationship between association bias and *early B* expression within conspecific male exposure (Table 4). However, with heterospecific male-exposure, we found contrasting linear relationships between association bias and *early B* expression. *Early B* encodes for early B-cell factor associated zinc finger transcription factor, functionally linked to lymphocyte regulation (Hagman *et al.* 1993; Lin & Grosschedl 1995) and olfactory neuron development (Wang *et al.* 1997). Although it is possible there may be some immune or olfactory requirements imposed to *G. affinis* females in the presence of heterospecific males, the up-regulation of *early B* may also represent part of a broad, transcriptome-level response in the *G. affinis* brain to the extreme heterospecific mating contexts (Fig. 2f).

The specific features of the different male phenotypes that trigger these contrasting neural responses are unknown. The large courting *P. latipinna* males in our experiment have large sail-like dorsal fins with conspicuous orange and iridescent blue coloration, while the more moderately sized coercive *P. latipinna* males lack such ornamentation. The two male phenotypes also differ in behavioral strategies with

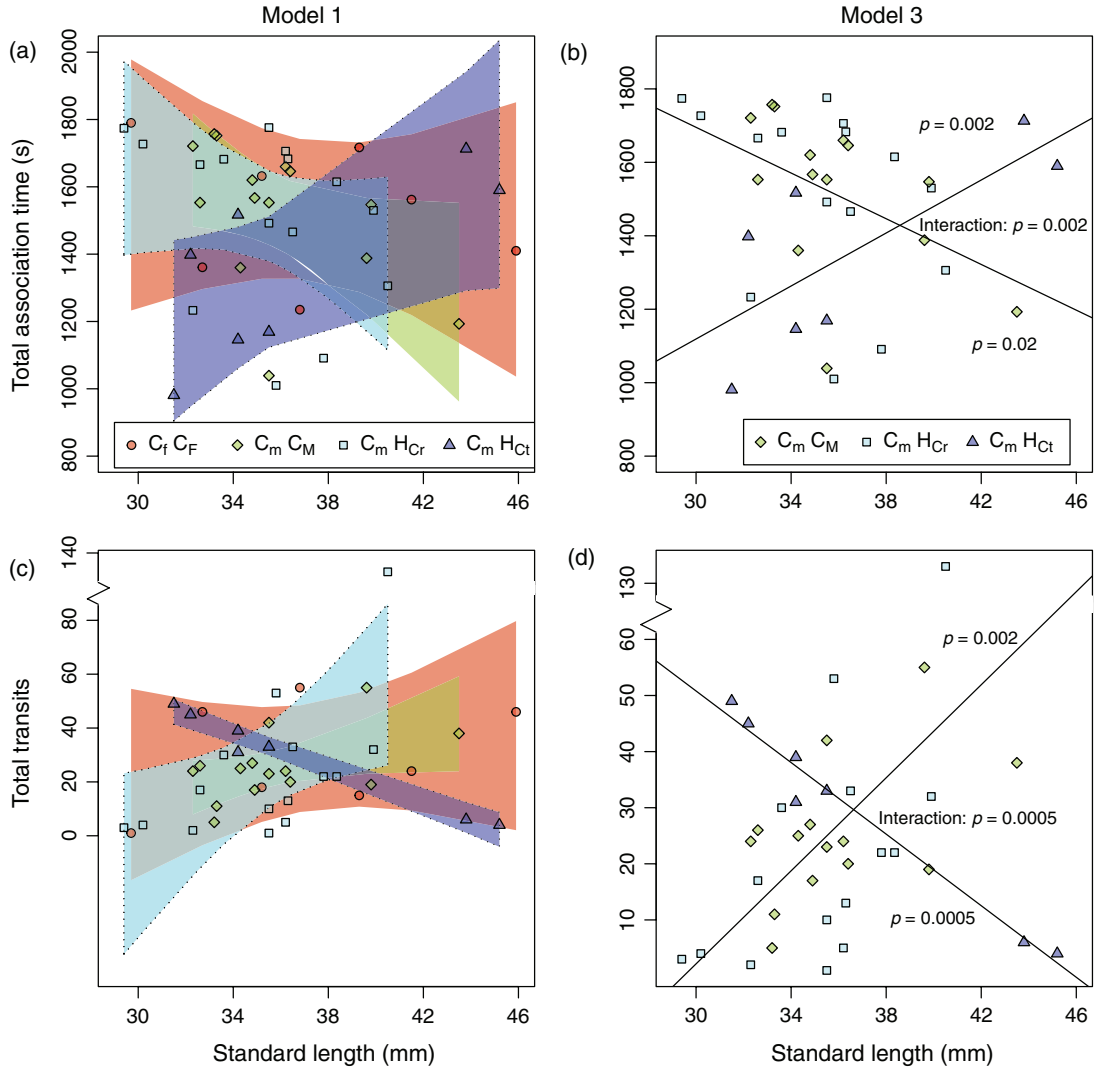

Figure 3: Female size (age/experience) predicts stimulus-specific behavior in *G. affinis* females exposed to courting and coercive males. Scatter plot with 95% confidence regions of generalized linear models of total association time (a) and number of total transits (c) against focal female standard length (SL) across each social treatment group: C_fC_F (red), C_mC_M (green), C_mH_{Cr} (light blue), C_mH_{Ct} (dark blue). Scatter plot of total association time (b) and total transits (d) by focal female SL in different male exposure contexts: pooled coercive male context (lighter regression line) including C_mC_M (green squares) and C_mH_{Cr} (light blue squares), and courting male context (darker regression line) represented by C_mH_{Ct} (dark blue triangles). Significant relationships are outlined by dashes in (a,c) and by solid regression lines in (b,d). See Tables 6–8 for statistical results.

the courting phenotype exhibiting more displays and less frequent coercive thrust than the coercive male *P. latipinna* (Becker *et al.* 2012; Travis & Woodward 1989). Whether the differential genetic response of females exposed to these two groups was triggered by ornamentation or behavioral differences is not known. Future work should try to tease apart the salient attributes that elicit the variation in synaptic plasticity gene responses in the brain.

Even though neuromolecular activity underlying the response to courting heterospecific signals in *G. affinis* females shares striking similarities to that of *X. nigrensis* in response to courting male conspecifics, we should be aware of potential differences. The presence of a courting heterospecific male recovered gene expression patterns associated with female choice in *G. affinis* females but this did not translate to a strong behavioral bias (here defined as >70% association bias) toward the courting male as is typically seen in *X. nigrensis* females exposed to a courting male phenotype (Cummings & Mollaghan 2006; Cummings *et al.* 2008; Wong *et al.* 2011). The lack of a strong behavioral bias when exposed to courting heterospecifics may be due to (1) species recognition, (2) lack of male courtship

Table 6: Regression models that predict total association time and total transit with standard length (SL) and treatment groups (Model 1: all social groups: C_fC_F, C_mC_M, C_mH_{Cr} and C_mH_{Ct}; Model 3: all male groups: C_mC_M, C_mH_{Cr} and C_mH_{Ct}). Analyses of variance of models that predict behavior by standard length across all social treatments (Model 1), and in male groups only (Model 2)

Model 1					
	Factors				
Response	Groups, $F(3, 35)$ (P)	SL, $F(1, 35)$ (P)	Groups × SL, $F(3, 25)$ (P)	$F(7, 35)$	P-value
Total association time (s)	1.11 (0.36)	0.66 (0.42)	3.75 (**0.02**)	2.18	0.06
Total transit	0.92 (0.44)	0.85 (0.36)	7.27 (**0.0006**)	3.63	**0.005**
Model 3					
	Factors				
Response	Groups, $F(1, 32)$ (P)	SL, $F(1, 32)$ (P)	Groups × SL, $F(1, 32)$ (P)	$F(3, 32)$	P-value
Total association time (s)	3.22 (0.08)	0.45 (0.5)	11.69 (**0.002**)	5.12	**0.005**
Total transit	0.25 (0.62)	1.75 (0.19)	14.89 (**0.0005**)	5.63	**0.003**

All the bolded P-values correspond to significant relationships that survived FDR correction.

Table 7: Regression coefficients (reflect the intercept or slope of the linear relationship between SL and behavior) for each group-specific predictors for Model 1

Model 1							
		Estimate (P-value)					
Response		C_fC_F	C_mC_M	C_mH_{cr}	C_mH_{ct}	R^2	R^2_{adj}
Total association time (s)	Intercept	1901.97 (**0.003**)	880.23 (0.33)	612.52 (0.48)	−1865.07 (0.03)		
	SL coefficient (slope)	−9.98 (0.53)	−25.07 (0.31)	−18.22 (0.45)	46.05 (**0.05**)	0.3	0.16
Total transit	intercept	−21.07 (0.71)	−29.24 (0.73)	−165.30 (0.05)	167.55 (**0.04**)		
	SL coefficient (slope)	1.35 (0.37)	0.76 (0.74)	4.64 (0.04)	−4.54 (0.04)	0.37	0.25

All the bolded P-values correspond to significant relationships that survived FDR correction.

activity toward heterospecific females, or (3) lack of female experience with courting phenotypes in the wild. In our study, we found no evidence for avoidance of heterospecific males in either the C_mH_{Cr} or C_mH_{Ct} treatments (Table 1), suggesting that females were not biasing their behavior due to species-recognition. Furthermore, the contrasting pattern of gene-by-behavior relationships (Fig. 2) evoked by different heterospecific phenotypes suggests that it is phenotypic recognition, rather than species recognition, that is driving these differences. Previous studies have shown that *P. latipinna* males showed strong discrimination against heterospecific females (Gabor & Ryan 2001; Ryan *et al.* 1996), so it is possible that the weak female behavioral response toward large *P. latipinna* males was due to limited male courtship. It is also possible that extended experience and physical interaction with courting males is required for females to exhibit strong behavioral preferences for this phenotype. Although the females from this study were drawn from an experimental population where *G. affinis* and *P. latipinna* occur in sympatry, our results may be driven by limited experience of *G. affinis* with courting *P. latipinna* phenotypes.

Table 8: Regression coefficients (reflect the intercept or slope of the linear relationship between SL and behavior) for each group-specific predictors for Model 3

Model 3					
		Estimate (P-value)			
Response		Coercive male	Courting male	R^2	R^2_{adj}
Total association time (s)	Intercept	2628.88 (**<0.0001**)	−2591.98 (**0.001**)		
	SL coefficient (slope)	−31.12 (**0.02**)	67.19 (**0.002**)	0.32	0.26
Total transit	Intercept	−122.19 (**0.008**)	268.67 (**0.0005**)		
	SL coefficient (slope)	4.15 (**0.002**)	−7.33 (**0.0005**)	0.35	0.28

All the bolded P-values correspond to significant relationships that survived FDR correction.

The contrasting expression patterns of a common set of genes associated with synaptic plasticity type processes may indicate a component of learning and memory in modulating appropriate female mating responses to male stimuli, and the divergent behavior outcomes may have been shaped by differential learning experience over the lifetime of females in coercive vs. mate choice taxa. In female-choice-dominated mating systems such as *X. nigrensis*, older and more experienced females exhibit stronger preferences than less experienced females for larger, courting males (Wong *et al.* 2011). In the current experiment, female *G. affinis* of the coercive mating system also changed the way in which they associate with the males as they age, becoming increasingly less likely to interact with coercive males (Fig. 3; Tables 6–8). Female *G. affinis* mate multiply and experience harassment from their conspecific males which can reduce female foraging efficiency (Pilastro *et al.* 2003) and cause injuries (Clark *et al.* 1954). Therefore it is not surprising that older females, who potentially experienced more conspecific harassments, are expected to associate less with coercive males. Experience may have warranted greater avoidance of these phenotypes.

It is intriguing that in the presence of a courting heterospecific male, older females showed an opposite pattern: associating longer with males and significantly lowering the number of exits (transits) from male association zones (Fig. 3). *G. affinis* females used in this study were sexually experienced and had previous exposure to heterospecific males (collected from a pond with mixed species population). Hence it is unclear whether older females are exhibiting differential behavioral responses due to previous experience with their own coercive male phenotypes, experience with heterospecific phenotypes, or a mixture of both. Nonetheless context-dependent female association response was amplified in older and more experienced females, which supports the concept of behavioral plasticity even in females of a mating system where mate choice is muted.

This study has shown that females in a coercive mating system modulate their whole-brain gene expression patterns based on the type of male with which they are interacting, and this plasticity is further tuned by age or experience. Our study suggests that such contrasting patterns of gene-by-behavior are responsive to available male phenotypes (courter vs. coercer), instead of being canalized within a species or mating system. This phenotypic plasticity at the level of the brain does not rule out a potential genetic basis, but rather characterizes the reaction norm of the neuromolecular response which is a key first step in understanding the genomic make-up of plastic traits (Aubin-Horth & Renn 2009). The transient neuromolecular activities that are discriminative to male mating type may contribute to long-term consequences for female mating decisions, and potentially the fitness of the two sexes. Flexibility in female preference response is the foundation of learning-based mate preference, which may influence the rate of mating character evolution more than sensory system bias (Price *et al.* 2003; Ryan & Cummings 2013; ten Cate & Rowe 2007). Further comparative investigation of the plastic and potentially learning-based mate choice mind can increase our understanding of the evolutionary origin and the contribution of female mate choice in mating system diversity.

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