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Mating evolution in *Gambusia* (Poeciliidae): an integration of behavior, molecules and morphology

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Mating evolution in *Gambusia* (Poeciliidae): an integration of behavior, molecules and morphology

by

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

The University of Texas at Austin

May 2014

Dedication

For Dahong Chen, the “lateral line” of my fish works.

Acknowledgements

I want to thank my advisor, Dr. Molly Cummings, for her intelligent and versatile guidance that made these three years colorful and fulfilled. Thank my collaborator and mentor, Dr. Mark Kirkpatrick, for his critics and encouragements loaded with knowledge, creativity and sense of humor. I also owe a great debt to my reader, Dr. Mike Ryan, who was always helpful for various interests I had, by being so honest while supportive, serious while vivacious. A special thank to Dr. Mary Ramsey, for being so sharp and kind; she graciously taught me molecular techniques and trouble shooted with me through various difficulties I encountered. I am grateful for my reader Dr. Hans Hoffman's support and the broad vision of behavior genomics that he has shared with us.

I thank my lab mates Chad Brock, Gina Calabrese, Laura Crothers, Ian Etheredge, Caitlin Friesen, Eben Gering, Kathleen Lynch, Luke Reding, Kat Ruddick, and Shulei Zhao for providing all kinds of helps and intriguing discussions that I needed over these years of my study.

I thank Minh Pham for data processing assistance and Dr. Trevor Price for helpful comments in Chapter 1; Dahong Chen, Pranav Dadhich, Korey Conley and Anton Pederson, along with Dr. Peter Dijkstra, for their large contributions to the study in Chapter 2. In addition, I thank the Mexican government for collecting permits (DGOPA07311-13709-2261), and Brackenridge Field Laboratory at the University of Texas at Austin for fish care facilities.
This thesis research was supported by NSF IOS-0843000 to MEC, grant DEB-0819901 to M.K and an EcoLab grant (3096781131) to SW.

Abstract

Mating evolution in *Gambusia* (Poeciliidae): an integration of behavior, molecules and morphology

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Female mate choice and male courtship display are critical behaviors for the understanding of character evolution driven by sexual selection. This thesis is designated to understand the evolutionary mechanism of these two behaviors with mosquito fish (*Gambusia*). In the first chapter, collaborated with Dr. Mark Kirkpatrick, we demonstrated positive coevolution of courtship display and morphological signatures of male coercion and male advantage in sexually antagonistic adaptation across 10 *Gambusia* species. This finding suggested that male display may have caused the evolution of morphologies involved in SAC, or conversely it may have evolved as a palliative byproduct of the morphologies. This unexpected observation raised new interpretation about evolutionary cause and consequence of displays across different mating systems. The second chapter examined whether neuromolecular underpinning of *G. affinis* female mate choice is canalized or plastic in mating systems that show variable extant of mate choice. With Dr. Mary Ramsey, we should 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 suggested that the

neuromolecular entities associated with female preference are plastic and responsive to different male phenotypes (courting or coercive) rather than a canalized response linked to mating system. Further, I proposed that female behavioral plasticity may involve learning because female association patterns shifted with experience/age. Compared to younger females, I find that more experienced females spend less time near coercive males but associate more with males in the presence of courters. We thus suggested a conserved learning-based neuromolecular process underlying the diversity of female mate preference across the mate choice and coercion-driven mating systems.

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Chapter 1[1]: Coevolution of male courtship and sexual conflict morphologies

INTRODUCTION

Sexually antagonistic selection has significant impact on mating character diversity (Parker, 1979; Wachtmeister & Enquist, 2000; Gavrilet, *et al.* 2000). In the coevolution of mating partners, advantages achieved by one generate selection for counteradaptations by the other (Holland & Rice, 1999; Rice, 1996; Arnqvist & Rowe, 2002; Lessells, 2006). Several morphological characters were found to evolve in sexually antagonistic coevolution (or SAC) (Gavrilet *et al.* 2000; Arnqvist & Rowe, 2005; Civetta & Clarke, 2000; Rice, 1996), but little is known about their evolutionary relation with mating characters that are not sexually conflicting. SAC is most often associated with taxa in which sperm competition drives an arms race between males and females over mating rate (Arnqvist & Rowe, 2002; Rice, 1996; Ross & Crews, 1977). Persistent escalation of SAC would lead to exaggerated offensive and defensive arms of the mating partners, which in turn incur extreme collateral damage and mating cost. A balancing mechanism is thus predicted to occur at high level of sexual conflict to bring SAC dynamic to equilibrium. Such balancing strategies may be embedded in mating characters that facilitates cooperation and alleviates bilateral sexually-conflicting adaptations.

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[1] Portions of this chapter has been submitted for publication.
Wang, S. M. T., Cummings, E. M. & Kirkpatrick, M. A. Coevolution of male courtship and sexual conflict characters in mosquitofish.

Contribution: Wang conjectured this study, collected data. Kirkpatrick suggested to model the behavior data with Discrete Markov Chain, wrote codes for it and analyzed data with Wang. Wang cond[1]ucted all the rest analyses. Wang, Cummings and Kirkpatrick interpreted results together. Wang wrote up the first manuscript draft for publication purpose. Wang, Cummings and Kirkpatrick edited the manuscript before submission.

Male display could be such a mating character. It is more commonly associated with taxa where females exert control over mating rate via female choice (Hebets, *et al.* 2011; Oliver & Lobel, 2013; Lenz, 1994; Gamble, *et al.* 2003). Male display may be beneficial to females or to both sexes as it can enable mate assessment (Andersson, 1994; Trivers, 1972), stimulate cooperation and parental investments after mating (Amstrong, 1963; Servedio, *et al.* 2013), and prime reproductive physiology (Bastock, 1967; Lehrman, 1959; Lehrman, 1964). On the other hand, display has also been proposed to be an exploitative behavior that fuels sexually antagonistic coevolution (Wachtmeister & Enquist, 2000; Holland & Rice, 1999).

Here we study the relationship between morphologies involved in SAC and male display in poeciliid fishes. Species in this clade have internal fertilization and gestation periods ranging from 20 to 30 days (Greven, 2011). Their mating systems fall into two general categories, male courtship and male coercion (Constantz, 1975). In species in which male display is predominant, males approach females slowly from the front and display immediately before copulation attempts (Liley, 1966; Constanz, 1989). In species in which male coercion is more common, males typically copulate by approaching females swiftly from behind, and displays are not as frequent (Holland & Rice, 1999; Constantz, 1975; Peden, 1972; Rosen & Tucker, 1961; Baerends et al. 1955; Constantz, 1975; Itzkowitz, 1971; Martin, 1975; McPhail, 1978; Parzefall, 1969; Farr, 1989).

In the genus *Gambusia*, mating is often coercive (Constantz, 1975; Peden, 1972; Farr, 1989), but some species do show male display even though the displays do not directly lead to copulation (Peden, 1970). Furthermore, there is evidence that morphological traits in males and females experience SAC (Peden, 1972; Constantz, 1984 Langerhans, 2011). During copulation,

males transfer sperm with the gonopodium, which is a modified anal fin. In some species the gonopodium has a rounded tip. In others, the tip is hooked and has claws, features that are thought to facilitate coercive copulation (Rosen & Tucker, 1961; Rosen & Gordon, 1953; Reynolds, *et al.* 1993; Greven, 2005). The hook and claw can facilitate sperm transfer (Kwan, *et al.* 2013) at the cost of female vaginal bleeding and injuries (Clark *et al.* 1954; Kadow, 1954), and these injuries might decrease the probability that the female will remate (Constantz, 1984). The female genital papilla is thought to coevolve with male gonopodium shape through a SAC process (Langerhans, 2011). The papilla is a pad-like structure at the opening of the oviduct. It is thought to deflect the gonopodium and give females more control over copulations (Constantz, 1984; Parzefall, 1973). Species with a pointed male gonopodium tend to have a large female papilla (Peden, 1972; Langerhans, 2011).

Previous work has suggested a relationship between the male gonopodium and display. Poeciliid species with long gonopodia generally display little (Rosen & Tucker, 1961), while species with short gonopodia tend to display more elaborately before copulation (Constantz, 1984). A quantitative analysis of this relationship is missing, however, and the involvement of other morphological features has not been examined.

These observations led us to investigate the coevolution between male display and morphologies associated with SAC in *Gambusia*. We study this question by examining the relationship between male display behavior and (i) male offensive morphology (gonopodium shape), and (ii) female defensive morphology (papilla size). Following Arnqvist and Rowe (2002), we assess the male morphological advantage using a ratio of the scores of the two structures.

To quantify the contribution of male display to mating, we analyzed data on behavioral sequences using an analytic method that calculates the rate of transition from a display to a copulatory thrust. A high transition rate occurs when display is closely followed by copulatory thrust, with few other intervening behaviors. We interpret this as representing a situation where male display plays an important role in mating. Phylogenetic analyses of these data suggest that male displays are more important when males have a morphological advantage over females that facilitates forced copulations.

METHODS

Male mating behavior

We obtained data on the mating behavior of 10 *Gambusia* species from the Ph.D. thesis of Alexander E. Peden (1970). He estimated the frequencies of transitions between pairs of the behaviors shown in Table 1.1. Full descriptions of the behaviors are given in the thesis.

Table 1.1 Gambusia male mating behaviors [36]. Behaviors in bold are the starting and ending states for DT rate.

Behavior	Description
1. Display	Side facing female, fins partly folded (frontal display); or swims parallel to female, fins extended (lateral display)
2. Others	Non-mating behaviors (e.g. feeding, sparring)
3. Orient	Faces towards female, straight-bodied, 2 to 10 cm from the female
4. Approach	Moves towards female from behind
5. Nibble	Gently contacts female body with mouth
6. Examine	Frequent pauses head at less than 1 cm from female genital opening
7. Swing	Swings gonopodium forward to a precopulatory position
8. Thrust	Propels body upward and directs gonopodium towards female genital area

To quantify the contribution of male display to mating, we determined the rate at which males proceed from a display (frontal and lateral displays; see Table 1.1) to a copulatory thrust. We first calculated the mean number of transitions between different behaviors that occur between a display and a copulatory thrust. The calculation, which uses Markov chain analysis, accounts for all of the infinite possible sequences of behaviors. A key assumption of the calculation is that the transitions are independent: the probabilities of transitions to the next behavioral state are influence by the current state but not by previous states. Details are given in the Appendix. Finally, we take the reciprocal of the mean number of transitions. We refer to this statistic as the display-to-thrust rate, or DT rate.

The DT rate measures the speed with which males pass from the display to a copulatory thrust. We interpret it to be a proxy for the relative importance of display to the copulation. A high DT rate suggests that there is a strong connection between display and copulation (Liley, 1966). A low DT rate, on the other hand, says that many behaviors intervene between a display and a copulation attempt, suggesting that display is less important.

The calculation of the DT rate was modified for four species. *G. atrora* has two types of displays, and for this species we averaged the DT rates starting with each of the displays. *G. vittata*, *G. gaigei* and *G. nobilis* were never observed to make a male display, and for these species we assigned a DT rate of 0 since clearly display is not important to copulation.

Female and male reproductive morphologies

We estimated the mismatch between female and male reproductive morphologies. We assessed male gonopodium shape using the categories defined by Peden (1972) and Langerhans

[38]: 0 = rounded, 1 = broadly acute, 2 = acute. We likewise used their categories for papilla size: 1 = papilla absent and aperture completely covered by tissue protuberance, 2 = papilla absent and aperture partially enclosed by external tissue, 3 = papilla small or absent, and aperture moderately sized, 4 = papilla small or moderate, and aperture large, 5 = papilla large and aperture large.

Our measure of the mismatch between female and male reproductive morphologies is the gonopodium-to-papilla ratio, or GP ratio, defined as: gonopodium shape score / papilla size score. A low GP ratio suggests that females have relatively more morphological control over copulations, while a large ratio suggests that males may be more effective at forcing copulations.

Statistical methods

We first tested for significant correlations between DT rate and the morphological measures in the absence of any phylogenetic correction. Because the scores for gonopodium shape and papilla size are not normally distributed, we assessed significance of these correlations using nonparametric statistics. We then controlled for possible phylogenetic effects in two ways. To assess the coevolution of the DT rate and the GP ratio, we used Felsenstein's independent contrasts method (Felsenstein, 1985) as implemented in Mesquite (Wayne & Maddison, 2011). This approach determines the correlation between evolutionary changes in the DT rate and the GP ratio without making assumptions about the direction of causality. Second, we used phylogenetic ANOVA (Garland *et al.* 1993) to determine if the DT rate depends on papilla size or gonopodium shape. These analyses assume that papilla size and gonopodium shape are

independent variables that evolve according to a Brownian motion model, and that DT rate is a dependent variable.

For the phylogenetically-corrected analyses, we used the phylogeny summarized by Langerhans (Langerhans, 2011). One clade of the phylogeny is a polytomy of four species. It can be resolved into a fully bifurcating tree in 15 ways. When testing for significance, we calculated the *p* value for each of these 15 resolutions, then averaged them to obtain an overall significance level.

RESULTS

Figure 1.1 shows Peden's (1970) transition matrix for *G. heterochir* in graphical form. Each node corresponds to a behavior listed in Table 1.1. The weight of an arrow connecting two nodes is proportional to the frequency of the transition out of the preceding behavior to the succeeding behavior. The DT rate is the average speed of progressing from a display (the triangular node) to a copulatory thrust (the square node). The DT rate of *G. heterochir* is around the median DT rate of the 10 species.

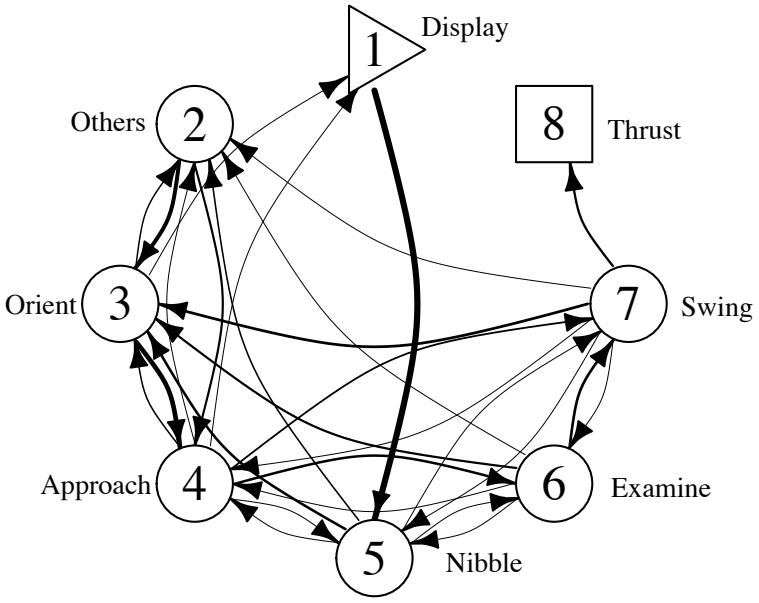

Figure 1.1 Mating behavior transition network of G. heterochir (data from Peden, 1970).

Figure 1.2 shows the graphs for the other nines species, ordered from low to high DT rate. The first three species (*G. nobilis*, *G. gaigei*, and *G. vittata*) have a DT rate of 0 because display was never observed. The highest DT rate is found in *G. geiseri*. It has a DT rate of 0.18, which implies that males pass through an average of 1/0.18 = 5.56 transitions from a display to a copulatory thrust. The DT rates, gonopodium shapes, papilla sizes, and GP ratios for all ten species are given in Table 1.2.

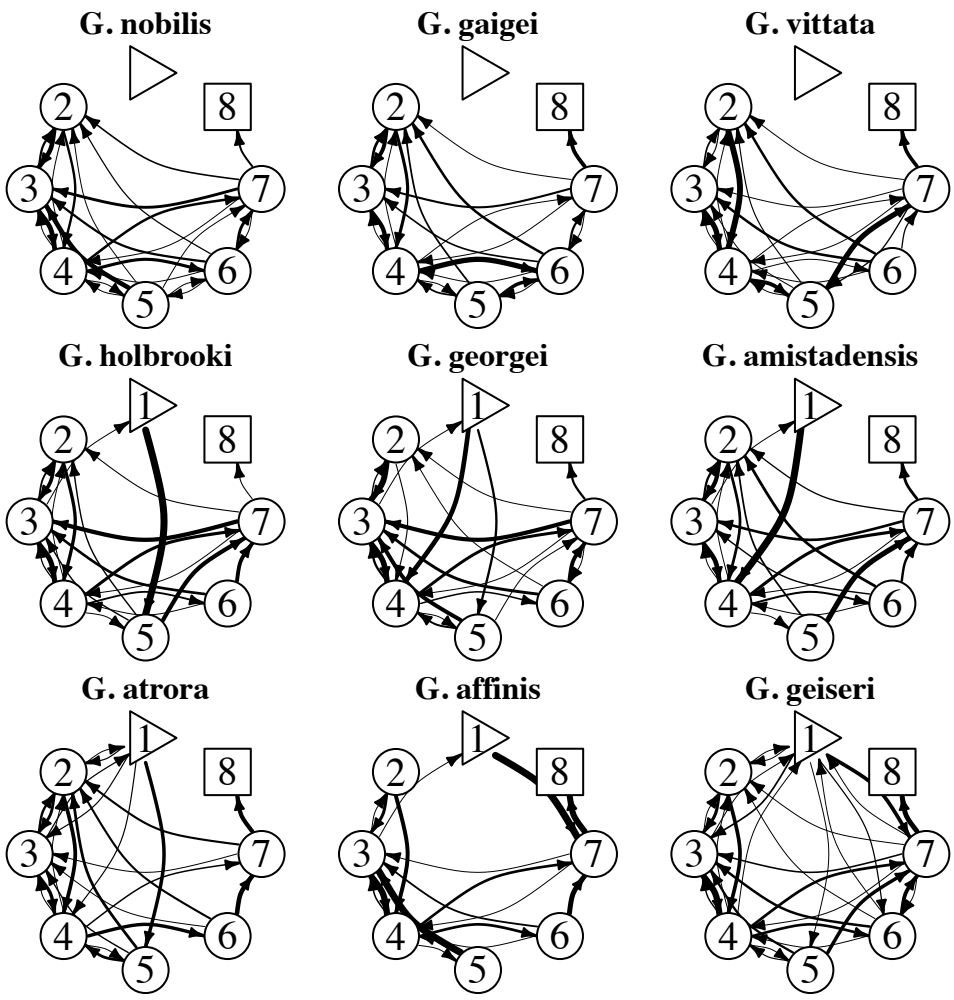

Figure 1.2 Male mating behavior transition rates for the other nine Gambusia species (data from Peden, 1970).

Table 1.2 The DT rate, male gonopodium shape score, female papilla size score, and GP ratio for the ten Gambusia species (data adapted from Langerhans, 2011).

Species	DT rate	Gonopodium	Papilla	GP ratio
G. nobilis	0.000	0	3	0.00
G. gaigei	0.000	1	3	0.33
G. vittata	0.000	0	1	0.00
G. holbrooki	0.028	2	5	0.40
G. heterochir	0.050	0	2	0.00
G. georgei	0.055	1	3	0.33
G. amistadensis	0.069	1	3	0.33
G. atrora	0.088	1	3	0.33
G. affinis	0.131	2	5	0.40
G. geiseri	0.176	2	5	0.40

We began by looking for relations between the morphological measures and DT rate before any corrections are made for possible phylogenetic effects. Figure 1.3 shows scatterplots of DT rate against papilla size and gonopodium shape. The correlation between DT rate and gonopodium shape is significant (Spearman's $\rho = 0.64$, $p = 0.048$). The correlation between DT rate and papilla size is also positive but not significant (Spearman's $\rho = 0.55$, $p = 0.099$). The correlation between DT rate and the GP ratio is positive and significant (Spearman's $\rho = 0.64$, $p = 0.048$).

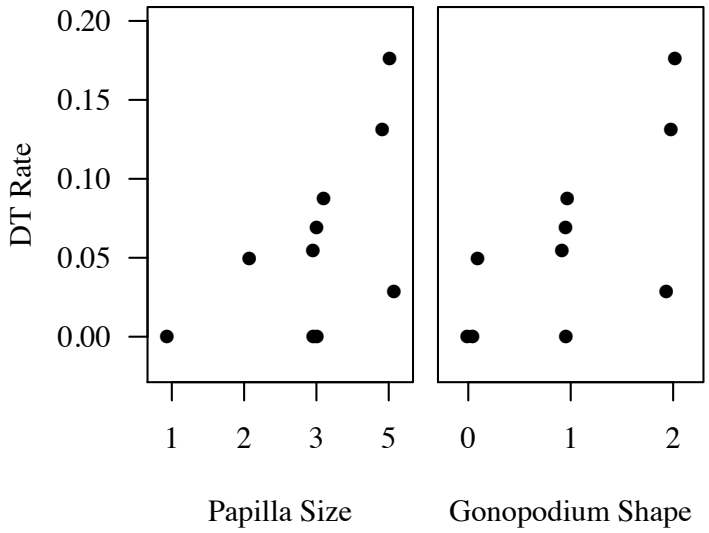

Figure 1.3 Scatterplots of DT rate against female papilla size (left; (Spearman's $\rho = 0.55$, p = 0.099) and male gonopodium shape (right; Spearman's $\rho = 0.64$, p = 0.048).

We then asked if there is evidence for coevolution after correcting for possible phylogenetic effects. Figure 1.4 maps the DT rate and the GP ratio onto the phylogeny. Figure 1.5 shows a scatterplot for the independent contrasts of those two measures. The correlation between them is significant ($r = 0.65$, $p = 0.03$). We also asked if DT rate shows a significant dependence on gonopodium shape and papilla size, again correcting for possible phylogenetic effects. The relation between DT rate and male gonopodium size is significant (Phylogenetic ANOVA, $p = 0.04$), but the relation between DT rate and female papilla size narrowly misses significance (Phylogenetic ANOVA, $p = 0.07$).

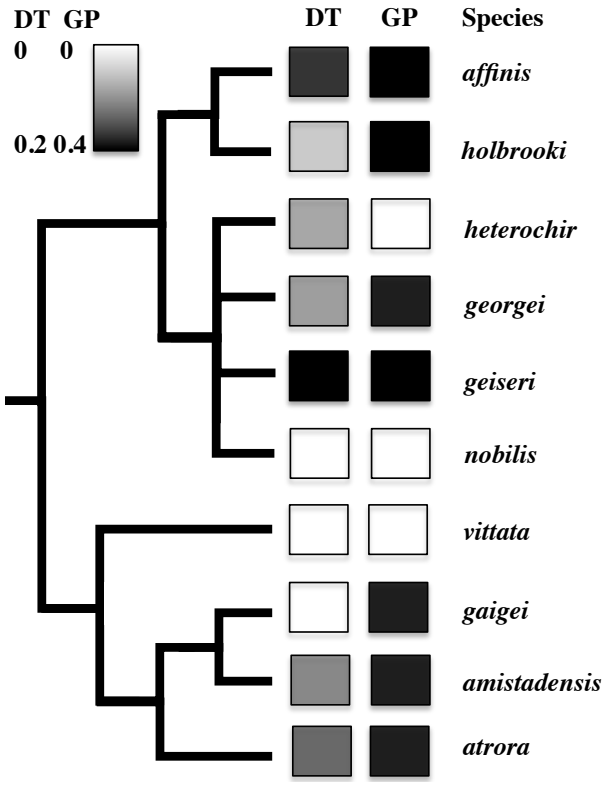

Figure 1.4 The phylogeny of the 10 Gambusia species (from [38]) showing their DT rates and GP ratios.

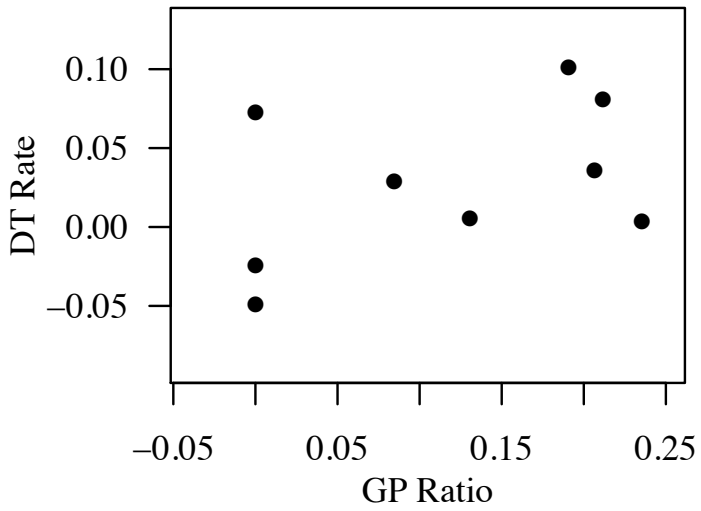

Figure 1.5 Scatterplot of the independent contrasts for DT rate and GP ratio (r = 0.65, p = 0.03).

DISCUSSION

We find a significant positive relationship in *Gambusia* between morphologies involved in sexually antagonistic coevolution (SAC) and the contribution of male display to mating. Specifically, there is a significant positive correlation between transition rate from display to copulatory thrust (the DT rate) and male morphology associated with SAC (hooks and claws on male gonopodia). There is also a significant positive correlation between the DT rate and the morphological advantage that males have over females (measured as the ratio of the gonopodia shape score to the female papilla size, the GP ratio). We propose two hypotheses to explain the coevolution of male display and male advantage in sexual conflict. In the first, display is the cause of the correlation, while in the second, evolution of morphologies involved in SAC lead to the evolution of display.

Display as an escalation of SAC

Male display may drive the positive correlation between *Gambusia* DT rate and morphological signatures of SAC (male gonopodial score and the GP ratio). Under this hypothesis, male display evolves to exploit a female receptivity response, which then causes the mating rate for females to reach a level above their optimum. That situation leads to the evolution of female behavioral or morphological defenses, followed by the evolution of greater male investment in morphological offenses. This idea is essentially Holland and Rice (1999) "chase-away selection" hypothesis (Holland & Rice, 1999; Arnqvist & Rowe, 2002). To date, there is little direct behavioral evidence for the evolution of female resistance to male displays. In poeciliids fishes, females of species with highly developed display (e.g. *Xiphophorus*

nigrensis) spend more time associating with courting males than do females from species with males that show display less frequently (e.g. *G. affinis*) (Lynch *et al.* 2012).

A second possibility is that male display mediates male-male competition. With high levels of male-male competition, male display may serve to signal potential rivals. In other species, male displays share elements with threat display (Fisher & Rosenthal, 2007; Borgia & Presgraves, 1998), suggesting that male display may have evolved from a competitive threat display. In the poecilliid *Xiphophorus birchmani*, the erect dorsal fin of the male lateral display is directed at competitors rather than females (Fisher & Rosenthal, 2007). If raised dorsal fins play the same role in *Gambusia*, then high DT rates may be correlated with strong male-male competition and elevated SAC.

Display as a de-escalation of SAC

The positive correlation between DT rate and morphologies involved in SAC may have evolved because display *results* from SAC rather than *drives* SAC. Under this hypothesis, male display serves to de-escalate SAC. The harm to females caused by coercive mating may also decrease the fitness of their mates. Poeciliids have internal fertilization, days to weeks of sperm storage before fertilization (Kobayashi & Iwamatsu, 2002), and 20-30 days gestation (Greven, 2011). Injury to females from mating therefore has abundant opportunities to decrease the male's fecundity. Male mating displays could lead to cooperative mating that increases the fitness of both partners. Lessells (Lessells, 2006) calls these "palliative traits".

For *Gambusia*, the male display may serve a palliative role, bringing both male and females to balance from the conflicting arm race in species with high levels of morphological SAC. It is

worth noticing that in the 'coercive only' *Gambusia*, males approach females from behind and attempt copulatory thrust without displaying at all (e.g *G. nobilis* and *G. vittata*). In species with rudimentary display (e.g. *G. affinis* and *G. geiseri*), males display just before a copulatory thrust. That may cause females to cooperate, and so make mating less harmful to both partners and also more successful.

Display may benefit males for three reasons. First, it can enhance the fertilization rate by inducing female cooperation (Condon & Wilson, 2006). More sperm are transferred in matings that are cooperative than forced (Philastro & Bisazza, 1999; Zimmerer & Kallman, 1989). In some taxa, display primes females physiologically for mating (Bastock, 1967; Lehrman, 1964). Second, display may increase male fitness by decreasing the harm to their mates that results from forced copulation. If forced copulation decreases the number of offspring that a female produces, there is an advantage to the male as well as the female to avoid that type of mating. Third, display might increase male reproductive success in the post-copulatory phase (Farr, 1989). Female poeciliids are most responsive to male display when their ova are most fertile (Liley, 1966; Farr, 1989; Kadow, 1954; Franck, 1964). Hence male display should also be selected if it increases male fertilization success (Farr, 1989).

Male display might benefit females as well as males. Display can allow females to assess potential mates prior to copulation. *Gambusia* females do in fact have mating preferences for male traits that may correlate with male quality. These traits include dark pigmentation (Gould, *et al.* 1999), which is associated with male performance in male-male competitions (Mcalister, 1958; Warburton, *et al.* 1957; Hubbs, 1969; Peden, 1973). Male display may therefore serve to increase female fitness by giving them cues about male quality. Thus at elevated intensity of

sexual conflict, display may emerge as a palliative trait to balance collateral harms by collateral benefits, thus secure both sexes from further escalation of costly conflict.

The evolution of display in poecilliids

The evolutionary trajectory of the rudimentary male display seen in *Gambusia* is not clear. Some workers have suggested that the ancestral state in poeciliids was coercive mating without a display (Farr, 1989; Ptacek & Travis, 1998), but that hypothesis has been questioned (Magurran, 2011). One possibility is that the ancestor of *Gambusia* had a more elaborate display that has now deteriorated. The sister clade of *Gambusia* is the monomorphic genus *Belonesox*, whose males have elaborate and prolonged display (Rosen & Tucker, 1961). Perhaps the ancestor of *Gambusia* and *Belonesox* had an elaborate display that has now been largely lost in *Gambusia* (Farr, 1989). Alternatively, the male display in *Gambusia* might currently be under selection to become more elaborate. For instance, *Poecilia* and *Gambusia* color elaboration tends to be more intense in populations under lower predation pressure (Godin & Briggs, 1996; Martin *et al.* 2013). Ecological factors surrounding poeciliid populations may explain the variable strength of selection for male display within and between lineages.

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Chapter 2[2]: Plasticity of the mate choice mind

INTRODUCTION

Female mate choice is an important aspect of sexual selection and mating system diversity (Darwin 1859; Guilford & Dawkins 1991). It is highly variable across mating systems: while it is usually muted in mating systems with male coercion as the predominant mating, highly distinctive in mating systems in which male courtship is the predominant mating strategy. Interestingly, the underlying neuromolecular response associated with mate choice also vary across mating systems (Lynch *et al.* 2012), yet little is known whether these responses are fixed (invariant with mating system) or plastic (variation due to environmental stimuli). For this interest, we asked if female 'choice-typical' behavioral and neural responses to males are constrained to 'choice' species or can be recovered it in a 'coercive' species with courting male stimuli?

In interesting system for this question is Poeciliidae, a family of livebearer fishes, demonstrating a spectrum of mating systems from female choice to male coercion (Farr 1989;

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[2] Portions of this chapter is now in press in the following article:

Silu M. T. Wang, Mary E. Ramsey and Molly E. Cummings* 2014 Plasticity of the mate choice mind: courtship evokes choice-like brain responses in females from a coercive mating system. Genes, Brain and Behavior. DOI: 10.1111/gbb.12124

Contribution: Cummings initially conjectured key ideas of this experiment and discussed with Wang. Wang and Cummings further completed experimental design and started behavioral components of this project. Wang finished the behavior tests and collected tissues for molecular study. Following the molecular protocol and supervision provided by Ramsey, Wang conducted molecular analysis of collected tissues. Then Wang did statistical analysis of this dataset wrote up the first manuscript draft. Wang, Ramsey and Cummings edited various versions of this manuscript before journal acceptance.

Bisazza 1993). 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 *Poeciliia 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 towards a favored male stimulus in female *X. nigrensis* (Cummings *et al.* 2008). In contrast, gene-behavior bias relationships show opposite trends in *G. affinis*, where females exhibit a negative correlation between genes associated with synaptic plasticity and strength of preference in the presence of coercive conspecific males (Lynch *et al.* 2012). These 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 mate choice-like positive 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 hypothesize 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.

METHOD

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; Wong, et al 2012; Ramsey, 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 of 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 versus large female conspecifics (C_fC_F, n=7), small versus large male conspecifics (C_mC_M, n=14), small conspecific versus coercive heterospecific males (C_mH_{Cr}, n=15), and small conspecific versus 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 minutes acclimatization period, and then released into the center compartment. This was followed by two 15-minute 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 minutes, 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 versus 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˚ Celsius with gentle shaking for 24 hours before the RNA later solution was taken out and brain tissue tubes immediately transferred to -80˚ Celsius storage until RNA extraction. The whole 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^{TM} 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 Appendix Table 2.5 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 Shapiroo-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 *neuroserpi*n 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:

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

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 & behavior

With the residuals of the three genes, we compared gene expression levels across different treatment groups with Kruskal-Wallis one-way 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 less than 0.05. Then 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 with response

variable (gene residual) and predictor variables (association bias and social treatment category). We developed two levels of analysis to evaluate the relationship between gene-by-behavior responses: 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 models.

Association of age & 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 social treatment groups) and Model 2 (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 ($p = 0.01$) but no difference in other conditions ($p > 0.05$).Comparing across male-exposed treatments, association bias, total association time, and total transits did not vary (Table1), even though there was a trend for greater association bias exhibited by focal females in C_mH_{Cr} conditions relative to the other male-exposure conditions (C_mC_M and C_mH_{Ct}) (Table1, Figure 2.1a).

Table 2.1 **a**, Association time (s, mean ± SD) of focal *Gambusia* females in each side of the association zone (with large or small stimulus) by treatment group. **b**, 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).

a,

	Association Time (s, mean ± SD)		Wilcoxon Signed Rank test	
Group	small stimulis	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

b,

	mean ± SD			Kruskal-Wallis one-way ANOVA		
Behaviors / Group	C_mC_M	C_mH_{Cr}	C_mH_{Ct}	*χ2*	df	*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

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 (Figure 2.1b, 1c, 1d; Kruskal-Wallis one-way ANOVA*, neuroserpin*: $p = 0.08$; *egr-1*: $p = 0.09$; *early B*: $p = 0.10$).

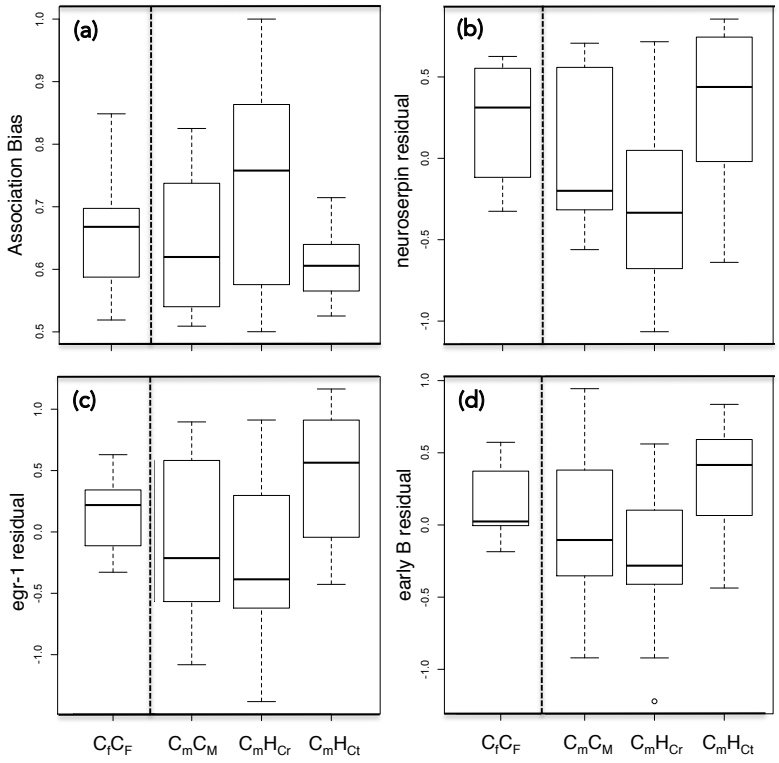

Figure 2.1. Boxplot of association bias (**a**) and *neuroserpin* (**b**) *egr-1* (**c**)_and *early-B* (**d**) residuals across large versus small female conspecifics group (C_fC_F) and male-exposure conditions: small versus large male conspecifics (C_mC_M), small conspecific male versus small coercive heterospecific male (C_mH_{Cr}) and small conspecific male versus large courting heterospecific male (C_mH_{Ct}). There is a trend of association bias difference across male-exposure conditions. There is a trend of differential expression of *neuroserpin*, *egr-1* and *early B* expression level across male-exposure contexts.

Context-specific correlations between gene expression and behavior

Modeling the relationship between gene expression and association bias revealed context-specific brain/behavior associations that were highly significant for females exposed to courter/coercive male pairs but only moderately predictive when general social groups (female controls) were included. Regression ANOVAs for Model 1 analyses (all social treatments included) indicated overall significance for *neuroserpin* and *egr-1* only (Table 2.2a). 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 2.2a). Regression models predicting the residuals of *neuroserpin*, *egr-1* and *early B* by association bias across all social treatments (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 2.2c, Fig 2.2a, 2.2c, 2.2e).

Table 2.2. The regression models that predicts 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}). **a**, **b**, Analyses of Variance of models that predict gene residuals by AB, across all social treatments (Model 1, **a**), and in heterospecific male groups (Model 2, **b**). **c**, **d**, regression coefficients (reflects the intercept or slopes 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 (**c**) and Model 2 (**d**). The bolded *p* values indicate significance that survived FDR correction.

a, ANOVA Model1

Response	Factors			F(7, 25)	*p*-value
(gene residual)	Groups, F(3, 35) (*p*)	Association bias, F(1, 35) (*p*)	Groups x Association bias, F(3, 35) (*p*)		
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

b, ANOVA Model2

Response	Factors			F (3, 18)	*p*-value
(gene residual)	Groups, F(1, 18) (*p*)	Association bias F(1, 18) (*p*)	Groups x Association bias F(1, 18) (*p*)		
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**

c, Model 1

Response		Estimate (*p*-value)				R^2	R^2_{adj}
(gene residual)		**C_fC_F**	**C_mC_M**	**C_mH_{Cr}**	**C_mH_{Ct}**		
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

d, Model 2

Response		Estimate (*p*-value)		R^2	R^2_{adj}
(gene residual)		**C_mH_{Cr}**	**C_mH_{Ct}**		
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

Examining the relationship between gene expression and association bias across male-exposed social contexts only (C_mC_M, C_mH_{Cr}, and C_mH_{Ct}) revealed no significance except for *early B* (*neuroserpin*: $p = 0.15$; *egr1*: $p = 0.27$, *early B*: $p = 0.04$). However, when we confine the analysis to only the heterospecific social groups (C_mH_{Cr}, and C_mH_{Ct}, Model 2) we found overall significance for all the three models (Table 2.2b). The residuals of *neuroserpin* and *early B* are significantly explained by association bias (Table 2.2b, $p < 0.05$), and the interaction between association bias and heterospecific male social group is a significant factor for all three genes (Table 2.2b, Figure 2.2b, 2d, 2f). In the presence of courting male heterospecifics (C_mH_{Ct}), association bias linearly predicted the residuals of *neuroserpin*, *egr-1* and *early B* (Table 2.2d, Figure 2.2b, 2.2d, 2.2f,). Meanwhile, in the presence of coercive male heterospecifics (C_mH_{Cr}), association bias only predicts the expression of *neuroserpin* and *early B* (Table 2.2d, Figure 2.2b and 2f,).

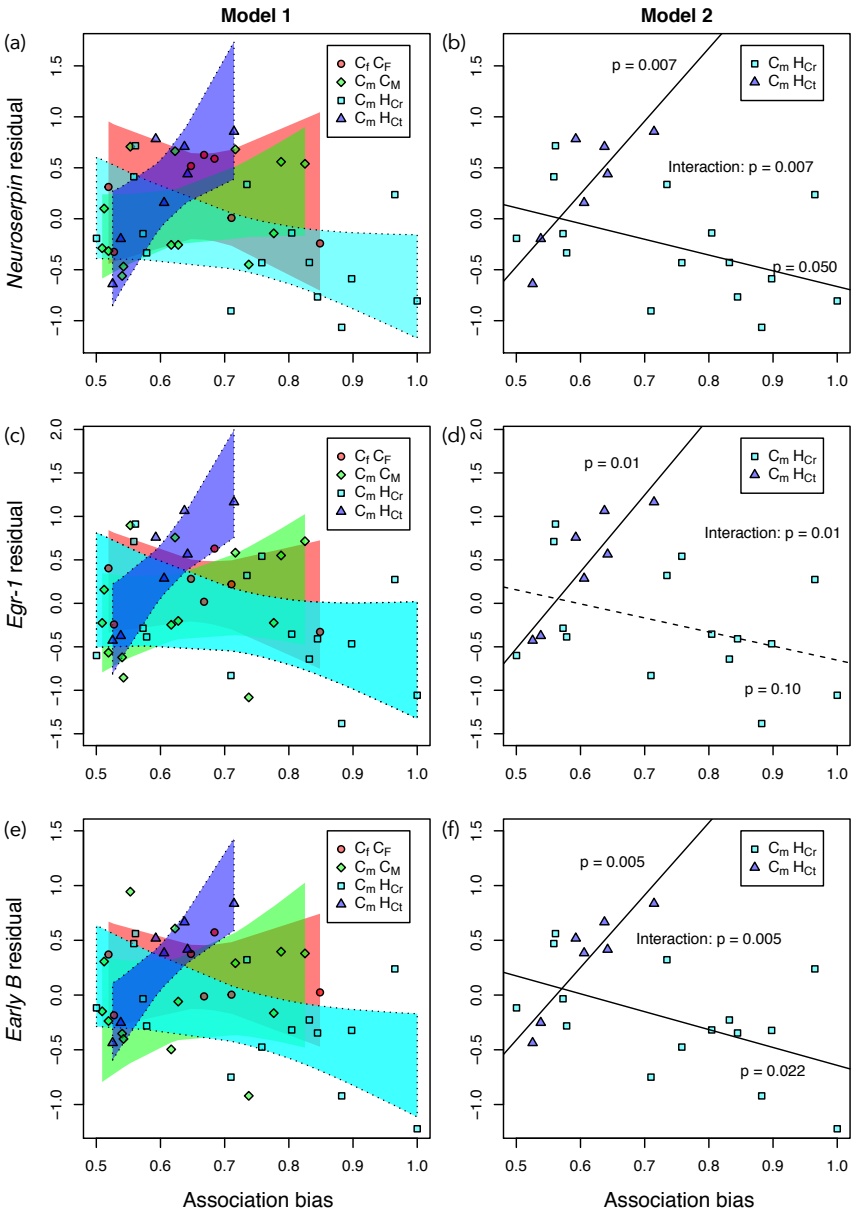

Figure 2.2. Scatter plot with 95% confidence regions of generalized linear models of gene residuals (**a**) *neuroserpin*, (**c**) *egr-1*, (**e**) *early B* and association bias across each social treatment group: C_fC_F (red), C_mC_M (green), C_mH_{Cr} (light blue), C_mH_{Ct} (dark blue). Scatter plot 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} (light blue squares), and C_mH_{Ct} (dark blue triangles). All models, except *early B*, were significant (see Table 2.2).

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 SL (a proxy of age) and association behaviors (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, $p = 0.06$) displayed by focal females across all the preference contexts. However, SL significantly predicts total transits exhibited by focal females (Table 2.3a, Figure 2.3c, $p = 0.01$). SL is the significant predictor for total transits in C_mH_{Cr}, and C_mH_{Ct} (Table 2.3c, $p < 0.05$, Figure 2.3c), but it significantly predicts total association time only within C_mH_{Ct} (Table 2.3c, $p < 0.05$, Figure 2.3a). The interaction between SL and social group significantly explained total transits and total association time (Table 2.3a, $p < 0.05$).

We then examined the effect of female age and/or experience on total association time and total transits within different male exposure groups (courting phenotypes (C_mH_{Ct}) versus coercive phenotypes (C_mC_M and C_mH_{Cr}) Model 2). ANOVAs for each response variable were significant (Table 2.3b), and revealed that both total transits (Figure 2.3d) and total association time (Figure 2.3b) are significantly predicted by the interaction between SL and male exposure (Table 2.3b, $p< 0.01$, Fig 3). Regression models show that SL is the significant predictor for total association time and total transit both within coercive (C_mC_M and C_mH_{Cr}) and courting (C_mH_{Ct}) male exposure groups, but in different directions (Figure 2.3b,d; $p < 0.05$).

Table 2.3. 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}). **a**, **b**, Analyses of Variance of models that predict behavior by standard length across all social treatments (Model 1, **a**), and in male groups only (Model 2, **b**). **c**, **d**, regression coefficients (reflects the intercept or slopes of the linear relationship between SL and behavior) for each group-specific predictors for Model 1 (**c**) and Model 3 (**d**). All the bolded *p* values correspond to significant relationships that survived FDR correction.

a, ANOVA Model 1

Response	Factors				
	Groups, F(3, 35) (*p*)	SL, F(1, 35) (*p*)	Groups x 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**

b, ANOVA Model 3

Response	Factors				
	Groups, F(1, 32) (*p*)	SL, F(1, 32) (*p*)	Groups x 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**

c, Model 1

Response		Estimate (*p*-value)					
		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

d, Model 3

Response		Estimate (p-value)			
		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

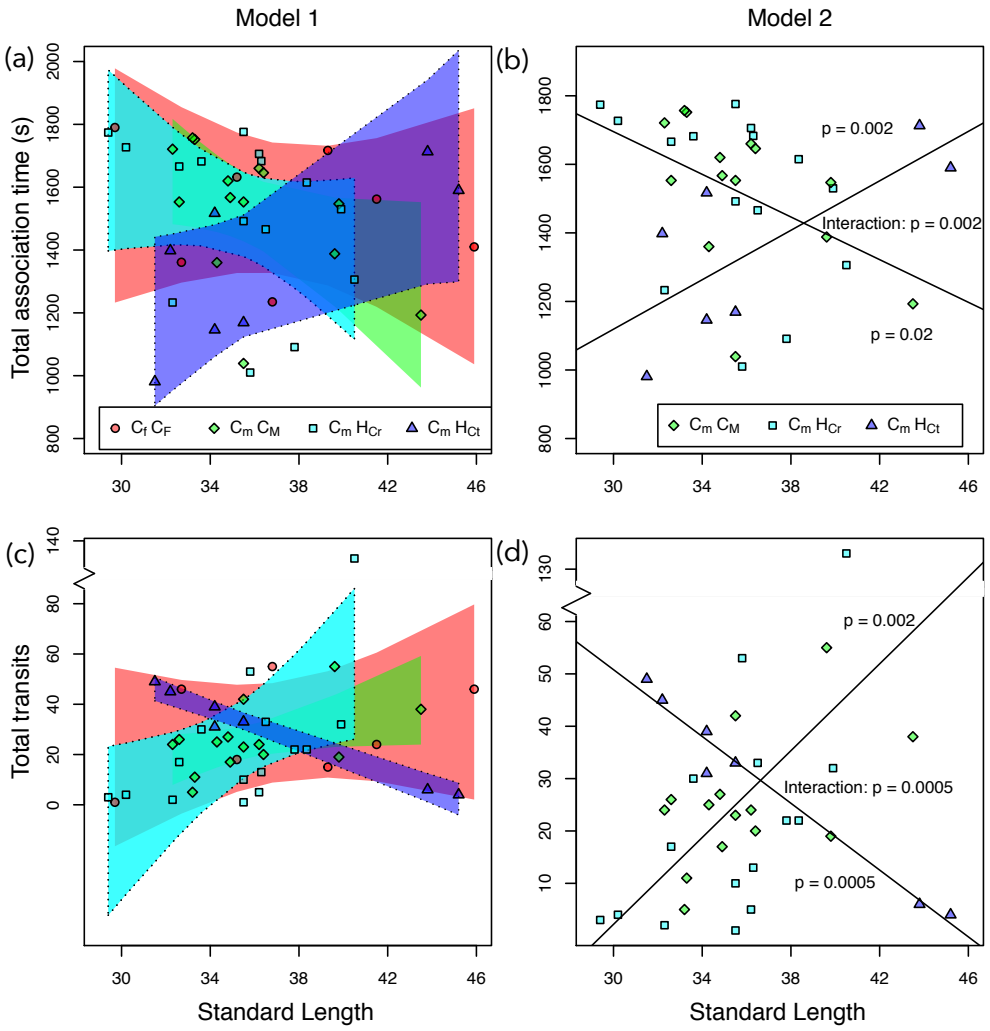

Figure 2.3. 95% confidence regions of generalized linear models of total association time (**a**) and number of total transits (**c**) against focal female standard length (SL) across each social treatment group: C_fC_F (red), C_mC_M (green), C_mH_{Cr} (light blue), C_mH_{Ct} (dark blue). Scatter plot 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). All models, except total association time, were significant (see Table 2.3).

DISCUSSION

Females avoid males in coercive mating systems, and this avoidance may be mediated in the brain via fixed neuromolecular responses that are invariant across variation in male phenotypes , or may be plastic and responsive to differences in male phenotypes. 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* (Figure 2.2) we observed (1) negative correlations between gene expression of all 3 genes examined and bias behavior with *coercive* males, as well as (2) positive correlations between these same genes and bias behavior with *courting* males (Figure 2.2, Table 2.2). 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 fixed neuromolecular processes intrinsic to 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 the current 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

(Figure 2.2 b, 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 a showy courting male. This current 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 pattern 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 towards 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 2.2c). 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 & Crosschedl 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 (Figure 2.2, f).

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 the courting phenotype exhibiting more displays and less frequent coercive thrust than the coercive male *P. latipinna* (Travis & Woodward 1989; Becker *et al.* 2012). 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) towards 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 (*i*) species recognition, (ii) lack of male courtship activity towards heterospecific females, or (*iii*) 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, suggesting that females were not biasing their behavior due to species-recognition. Furthermore, the contrasting pattern of gene-by-behavior relationships (Figure 2.2) evoked by different heterospecific phenotypes suggests that it is phenotypic recognition, rather than species recognition, that is driving these differences, although the contribution of previous sexual and social experience in shaping the female's brain/behavior response should not be underestimated. Previous studies have shown that *P. latipinna* males showed strong discrimination against heterospecific females (Ryan *et al.* 1996; Gabor & Ryan 2001), so it is possible that the weak female behavioral response towards 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.

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

versus 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 (Figure 2.3; Table 2.3). Female *G. affinis* mate multiply and experiences harassment from their conspecific males, which can reduce female foraging efficiency (Pilastro *et al.* 2003) and cause injuries (Clarke *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 from male association zones (Figure 2.3). *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 versus coercer), instead of being fixed within a species or mating system. This phenotypic plasticity at the level of the brain does not rule out a potential genetic basis of such plasticity, 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 (ten Cate & Rowe 2007; Price *et al.* 2003; Ryan & Cummings 2013). 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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Appendix

1. Markov Chain analysis of *Gambusia* mating behavior matrices.

Introduction

This notebook calculates "DT rate" during courtship in mosquitofish (genus *Gambusia*). DT rate is a measure of the speed with which fish proceed from an initial display to a copulatory thrust. We define DT rate as the reciprocal of the average number of steps taken in a Markov chain that describes the transitions between behavioral states. The behavioral states, and the frequencies of transitions between them, are from Peden's thesis (1972, University of Texas at Austin).

Calculations

The expected number of steps to reach an absorbing state (thrust, in our context) is a standard problem in the analysis of Markov chains. We number the behavioral states (1, 2, ..., n), where State 1 is the initial display and State n is a copulatory thrust. For an individual in state i, the probability that it is next in state j is written as p_{ij}. The number of steps that it takes to go from state i to a thrust (which is a random variable) is t_i. We can write that as

$$t_i = 1 + \sum_{j<n} p_{ij}\, t_j, \tag{1}$$

where we define $p_{ii} = 0$. The first quantity on the right is 1, which simply appears because i is at least 1 step away from j. The summation accounts for all the next states that the individual could assume after leaving state i. If it goes to state j, then the number of steps it will now take to a thrust is t_j.

Our goal is to find the average number of steps to go from i to a thrust, which we denote as $\bar{t}_i$. We begin by taking the expectation of both sides of equation (1). The result looks very similar, but with t_i replaced by $\bar{t}_i$. The system of equations for the $\bar{t}$ can be written in matrix form as

$$\bar{\mathbf{t}} = \mathbf{1} + \mathbf{P}\,\bar{\mathbf{t}}, \tag{2}$$

where $\bar{\mathbf{t}}$ is the vector of the $\bar{t}_i$, $\mathbf{P}$ is the matrix of the p_{ij} (with $i, j < n$), and $\mathbf{1}$ is a vector of 1s. (Note that $\mathbf{P}$ is not a stochastic matrix: the rows to not sum to unity because those elements don't include the probability that a state is followed by thrust.)

The solution for the expected number of steps from each of the states to a thrust is found simply by rearranging Equation (2):

$$\bar{\mathbf{t}} = (\mathbf{I} - \mathbf{P})^{-1}\,\mathbf{1}. \tag{3}$$

The DT rate is then:

$$\text{DT rate} = 1 / \bar{t}_1 .$$

An example

Take the case of three states, where the initial display is State 1 and the copulatory thrust is State 3. The vector of expected number of steps to reach thrust from any of the other behavioral states is is:

$$\bar{\mathbf{t}} = \{\bar{t}_1, \bar{t}_2\}$$

The matrix of transition probabilities is:

$$\mathbf{P} = \begin{pmatrix} 0 & p_{1,2} \\ p_{2,1} & 0 \end{pmatrix}$$

The solution for $\bar{\mathbf{t}}$ is:

```
P = Table[p_{i,j}, {i, 2}, {j, 2}] /. p_{i_,i_} → 0;
One = Table[1, {i, 2}];
Id = IdentityMatrix[2];
Inverse[Id - P].One // Simplify
```

$$\left\{\frac{1 + p_{1,2}}{1 - p_{1,2}\, p_{2,1}}, \frac{1 + p_{2,1}}{1 - p_{1,2}\, p_{2,1}}\right\}$$

Thus we find that

$$\text{DT rate} = 1/\bar{t}_1 = \frac{1 - p_{12}\, p_{21}}{1 + p_{12}\, p_{21}}$$

Data analyses

In this section we calculate the DT rates.

- **Preliminaries**

The behavioral states are:

1) initial display
2) non-courtship
3) orient
4) approach
5) nibble
6) examine
7) swing
8) thrust

(An exception to this list occurs with *G. atrora*, where there are two types of initial displays. Details are given below.)

The following routines are used in the analyses.

This function converts Peden's count data into our format: the behaviors are numbered as shown above, and c_{ij} is the number of times that behavior *i* was followed by behavior *j*. The matrices with these counts have dimension [7 x 8] (because we are not interested in transitions after we reach State 8).

```
cMaker[countMat_] := Module[
  {k = {3, 1, 2, 4, 5, 6, 7, 8}},
  Table[countMat[[k[[i]], k[[j]]]],
    {i, 8}, {j, 7}] // Transpose
 ]
```

```
tBar[cMat_] := Module[
  {nStates = (cMat // Length), P, IMat, vec1, tBar, tVec},
  P = Table[cMat[[i, j]] / Total[cMat[[i]]] // N, {i, nStates}, {j, nStates}];
  Do[P[[i, i]] = 0, {i, nStates}] ;
  IMat = IdentityMatrix[nStates];
  vec1 = Table[1, {i, nStates}];
  tBar = Inverse[IMat - P].vec1;
  tVec = Table[t̄ᵢ, {i, nStates}];
  Table[Print[tVec[[i]], " = ", tBar[[i]]], {i, nStates}];
 ]
```

■ Toy examples

To illustrate how things work, let's try a couple of toy examples.

■ Example 1

In this example, behavior 1 and behavior 2 are always followed by behavior 3. The average number of steps that it takes to reach behavior 3 must therefore be $\bar{t}_1 = \bar{t}_2 = 1$.

Here is the matrix where c_{ij} is the number of times behavior i was followed by behavior j. Notice that there are 2 rows (corresponding to the two possible initial states) and three columns (corresponding to the two initial states plus the final state):

```
cToy1 =
  {{0, 0, 1},
   {0, 0, 1}};
```

Here are the expected number of steps:

```
cToy1 // tBar
```

$\bar{t}_1$ = 1.

$\bar{t}_2$ = 1.

That looks good.

■ Example 2

In this example, behavior 1 is followed by behavior 2 and behavior 3 with equal probability. Likewise, behavior 2 is followed by behavior 1 and behavior 3 with equal probability. The average number of steps that it takes to reach behavior 3 is $\bar{t}_1 = \bar{t}_2 = 2$.

Here is the matrix. Notice that there are 2 rows (corresponding to the two possible initial states) and three columns (corresponding to the two initial states plus the final state):

```
cToy2 =
  {{0, 1, 1},
   {1, 0, 1}};
```

Here are the expected number of steps:

```
cToy2 // tBar
```

$\bar{t}_1$ = 2.

$\bar{t}_2$ = 2.

That looks good.

■ Example 3

The average number of steps depends on the probabilities of transitions. Thus the values of $\bar{t}$ are unaffected if the matrix of c_{ij} is multiplied by a constant.

Here is a matrix that is the one used in example 2, but multiplied by 5:

```
cToy3 =
  {{0, 5, 5},
   {5, 0, 5}};
```

Here are the expected number of steps:

```
cToy3 // tBar
```

$\bar{t}_1 = 2.$

$\bar{t}_2 = 2.$

That looks good: the results are the same as for example 2.

- **Examples 4 & 5**

A simple example of a network that shows "courtship" behavior is this one. State 1 is the display, State 2 is some other behavior, and State 3 is copulation.

```
cToy4 =
  {{0, 1, 1},
   {1, 0, 0}};
```

```
cToy4 // tBar
```

$\bar{t}_1 = 3.$

$\bar{t}_2 = 4.$

```
dt4 = 1 / 3.
```

```
0.333333
```

Now consider a network that shows "coercion": you can only get from the initial display (state 1) to copulation (state 3) by doing some other behavior (state 2):

```
cToy5 =
  {{0, 1, 0},
   {1, 0, 1}};
```

```
cToy5 // tBar
```

$\bar{t}_1 = 4.$

$\bar{t}_2 = 3.$

```
dt5 = 1 / 4.
```

```
0.25
```

Comparing examples 4 and 5, we see that for these transition matrices, the "courtship" network gives a higher transition rate from initial display to copulation.

Now consider this other example of a courtship network:

```
cToy6 =
  {{0, 6, 1},
   {1, 0, 0}};
```

```
cToy6 // tBar
```

$\bar{t}_1 = 13.$

$\bar{t}_2 = 14.$

The DT rate from initial courtship to copulation is:

```
1 / 13.
```

```
0.0769231
```

We see that the DT rate is now *lower* than for the coercive network. That's because the sequences leading to copulation spend many steps making transitions between state 1 and state 2 before reaching state 3.

The bottom line: it is not just the topology of the network (for example, whether there is a direct connection from initial display to final copulation) that determines DT rate.

■ *Gamusia affinis*

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior i was followed by behavior j:

```
cAff =
  {{0, 0, 0, 0, 0, 0, 3, 0},
   {0, 0, 7, 9, 0, 0, 0, 0},
   {3, 6, 0, 75, 0, 0, 0, 0},
   {0, 0, 20, 0, 1, 35, 34, 0},
   {0, 0, 1, 0, 0, 0, 0, 0},
   {0, 0, 10, 1, 0, 0, 27, 0},
   {0, 3, 31, 2, 0, 0, 0, 30}};
```

```
cAff // tBar
```

$\overline{t}_1$ = 7.63616

$\overline{t}_2$ = 10.7692

$\overline{t}_3$ = 10.3566

$\overline{t}_4$ = 9.3124

$\overline{t}_5$ = 11.3566

$\overline{t}_6$ = 8.68565

$\overline{t}_7$ = 6.63616

The DT rate is:

```
dtAff = 1 / 7.636
```

```
0.130959
```

■ *G. amistadensis*

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior i was followed by behavior j:

```
cAmi =
  {{0, 0, 0, 1, 0, 0, 0, 0},
   {0, 0, 28, 18, 0, 0, 0, 0},
   {1, 19, 0, 39, 0, 0, 0, 0},
   {0, 4, 8, 0, 5, 18, 28, 0},
   {0, 1, 0, 0, 0, 0, 3, 0},
   {0, 8, 4, 1, 0, 0, 7, 0},
   {0, 7, 14, 1, 0, 0, 0, 15}};
```

```
cAmi // tBar
```

$\bar{t}_1$ = 14.4136

$\bar{t}_2$ = 15.4274

$\bar{t}_3$ = 15.0791

$\bar{t}_4$ = 13.4136

$\bar{t}_5$ = 12.347

$\bar{t}_6$ = 14.3528

$\bar{t}_7$ = 9.98683

The DT rate is:

```
dtAff = 1 / 14.41
```

0.0693963

▪ *G. atrora*

This species differs from the other: there are two types of initial display (frontal and lateral). We will calculate the DT rate for this species as the average rate starting from each of those initial conditions.
The behavioral states are:

1) frontal display
2) lateral display
3) non-courtship
4) orient
5) approach
6) nibble
7) examine
8) swing
9) thrust

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior *i* was followed by behavior *j*:

```
cAtr =
  {{0, 0, 0, 1, 0, 3, 0, 0, 0},
   {0, 0, 1, 0, 1, 0, 0, 0, 0},
   {0, 2, 0, 16, 21, 0, 0, 0, 0},
   {4, 0, 8, 0, 11, 0, 0, 0, 0},
   {0, 0, 3, 5, 0, 2, 16, 7, 0},
   {0, 0, 2, 1, 2, 0, 0, 0, 0},
   {0, 0, 6, 1, 0, 0, 0, 13, 0},
   {0, 0, 6, 2, 0, 0, 0, 0, 12}};
```

The expected numbers of steps to reach thrust are:

```
cAtr // tBar
```

$\bar{t}_1 = 10.7213$

$\bar{t}_2 = 12.1338$

$\bar{t}_3 = 12.0334$

$\bar{t}_4 = 11.9448$

$\bar{t}_5 = 10.2343$

$\bar{t}_6 = 12.296$

$\bar{t}_7 = 8.9802$

$\bar{t}_8 = 5.80451$

The DT rate is:

$$\text{dtAtr} = \frac{1}{2}\left(\frac{1}{10.72} + \frac{1}{12.13}\right)$$

```
0.08786190646109929
```

■ *G. gaigei*

Initial displays were not observed in this species. It was assigned a DT rate of 0.

■ *G. geiseri*

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior *i* was followed by behavior *j*:

```
cGei =
 {{0, 1, 11, 0, 1, 8, 24, 0},
  {1, 0, 17, 20, 0, 0, 0, 0},
  {18, 15, 0, 260, 0, 0, 0, 0},
  {3, 2, 65, 0, 4, 118, 122, 0},
  {0, 0, 2, 1, 0, 0, 3, 0},
  {4, 5, 50, 4, 0, 0, 76, 0},
  {3, 2, 51, 10, 0, 2, 0, 159}}
```

The expected numbers of steps to reach thrust are:

```
cGei // tBar
```

$\bar{t}_1 = 5.67504$

$\bar{t}_2 = 7.46365$

$\bar{t}_3 = 7.0552$

$\bar{t}_4 = 6.00027$

$\bar{t}_5 = 5.87244$

$\bar{t}_6 = 5.80518$

$\bar{t}_7 = 3.04133$

The DT rate is:

$\bar{t}_1$ = 5.67504

$\bar{t}_2$ = 7.46365

$\bar{t}_3$ = 7.0552

$\bar{t}_4$ = 6.00027

$\bar{t}_5$ = 5.87244

$\bar{t}_6$ = 5.80518

$\bar{t}_7$ = 3.04133

The DT rate is:

```
dtGei = 1 / 5.675
```

0.176211

```
0.1762114537444934`
```

0.176211

■ *G. georgei*

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior i was followed by behavior j:

```
cGeo =
  {{0, 0, 0, 2, 1, 0, 0, 0},
   {0, 0, 54, 10, 0, 0, 0, 0},
   {3, 42, 0, 170, 0, 0, 0, 0},
   {0, 0, 64, 0, 6, 42, 82, 0},
   {0, 0, 4, 2, 0, 0, 1, 0},
   {0, 1, 18, 0, 0, 0, 24, 0},
   {0, 3, 63, 5, 0, 1, 0, 34}};
```

The expected numbers of steps to reach thrust are:

```
cGeo // tBar
```

$\bar{t}_1$ = 18.239

$\bar{t}_2$ = 19.0212

$\bar{t}_3$ = 18.2486

$\bar{t}_4$ = 16.7932

$\bar{t}_5$ = 18.1305

$\bar{t}_6$ = 16.5226

$\bar{t}_7$ = 13.3322

The DT rate is:

```
dtGeo = 1 / 18.24
```

0.0548246

$\bar{t}_1$ = 20.1322

$\bar{t}_2$ = 19.3486

$\bar{t}_3$ = 18.8697

$\bar{t}_4$ = 17.4009

$\bar{t}_5$ = 19.1322

$\bar{t}_6$ = 17.252

$\bar{t}_7$ = 12.7242

The DT rate is:

```
dtHet = 1 / 20.13
```

0.0496771

■ *G. holbrooki*

This is the matrix with the counts of transitions observed, where c_{ij} is the number of times behavior *i* was followed by behavior *j*:

```
cMye =
  {{0, 0, 0, 0, 1, 0, 0, 0},
   {0, 0, 29, 26, 0, 0, 0, 0},
   {1, 27, 0, 75, 0, 0, 0, 0},
   {0, 8, 20, 0, 4, 29, 49, 0},
   {0, 1, 1, 0, 0, 0, 3, 0},
   {0, 0, 11, 2, 0, 0, 16, 0},
   {0, 10, 40, 6, 0, 0, 0, 10}};
```

```
cMye // tBar
```

$\bar{t}_1$ = 35.2168

$\bar{t}_2$ = 36.1731

$\bar{t}_3$ = 35.8698

$\bar{t}_4$ = 34.396

$\bar{t}_5$ = 34.2168

$\bar{t}_6$ = 34.2728

$\bar{t}_7$ = 31.347

The DT rate is:

```
dtMye = 1 / 35.22
```

0.028393

■ *G. nobilis*

Initial displays were not observed in this species. It was assigned a DT rate of 0.

- ***G. vittata***

Initial displays were not observed in this species. It was assigned a DT rate of 0.

Table 2.4 Regression models predict gene (*neuroserpin*, *egr-1* and *early B*) residuals with TAT (total association time) and social exposure (Model 1: C_fC_F, C_mC_M, C_mH_{cr}, and C_mH_{ct}; Model 2: C_mH_{cr} and C_mH_{ct}). (**a**) Regression coefficient (intercept or slopes of the linear relationship between TAT and gene residuals) and *p* values for each group-specific predictor, along with the overall R^2 and R^2-adjusted. (**b**) Analyses of Variance of the six models shown in (**a**). All genes exhibited overall significance for the model 2 analysis (male heterospecific groups only; $p < 0.05$).

(a)

			Estimate (*p*-value)				Overall	
Response			**C_fC_F**	**C_mC_M**	**C_mH_{cr}**	**C_mH_{ct}**	R^2	R^2_{adj}
neuroserpin residual	Model 1:	Intercept	2.33 (0.13)	-1.45 (0.18)	-1.29 (0.46)	-0.71 (0.70)		
	All social groups	TAT coefficient (slope)	-0.001 (0.17)	0.001 (0.21)	0.0005 (0.64)	0.0004 (0.74)	0.31	0.17
	Model 2:	Intercept			1.24 (0.23)	0.43 (0.67)		
	Heterosp. Male groups	AB coefficient (slope)			-1.57 (0.13)	-0.11 (0.91)	0.36	0.26
egr-1 residual	Model 1:	Intercept	1.37 (0.47)	-1.71 (0.45)	0.30 (0.89)	0.37 (0.87)		
	All social groups	TAT coefficient (slope)	-0.0008 (0.51)	0.001 (0.49)	-0.0005 (0.74)	-0.0002 (0.92)	0.25	0.1
	Model 2:	Intercept			1.67 (0.12)	-0.07 (0.97)		
	Heterosp. Male groups	TAT coefficient (slope)			-0.001 (0.07)	0.0003 (0.80)	0.37	0.26
early B residual	Model 1:	intercept	0.98(0.50)	-1.20(0.49)	0.20 (0.90)	0.33 (0.85)		
	All social groups	TAT coefficient (slope)	-0.005(0.57)	0.0007(0.54)	-0.0004 (0.71)	-0.0002 (0.86)	0.26	0.12
	Model 2:	Intercept			1.18 (0.14)	0.12 (0.92)		
	Heterosp. Male groups	TAT coefficient (slope)			-0.0009 (0.08)	0.0002 (0.82)	0.38	0.27

(b)

		Factors			Overall	
Response		Groups	Total association time	Groups x Total association time	F	*p*-value
neuroserpin residual	Model1:	F (3, 35) = 2.97	F (1, 35) = 4.23	F (3, 35) = 0.77	F (7, 25) =	
	All social groups	***p* = 0.04**	***p* = 0.05**	*p* = 0.52	2.21	0.06
	Model2:	F (1, 18) = 6.09	F (1, 18) = 4.09	F (1, 18) = 0.01	F(3, 18) =	
	Heterosp. Male groups	***p* = 0.02**	*p* = 0.06	*p* = 0.91	3.4	**0.04**
egr-1 residual	Model1:	F (3, 35) = 2.16	F (1, 35) = 3.27	F (3, 35) = 0.72	F (7, 25) =	
	All social groups	*p* = 0.11	*p* = 0.08	*p* = 0.55	1.7	0.14
	Model2:	F (1, 18) = 5.77	F (1, 18) = 4.63	F (1, 18) = 0.07	F(3, 18) =	
	Heterosp. Male groups	*p* = 0.03	***p* = 0.05**	*p* = 0.80	3.49	**0.04**
early B residual	Model1:	F (3, 35) = 2.54	F (1, 35) = 2.96	F (3, 35) = 0.67	F (7, 25) =	
	All social groups	*p* = 0.07	*p* = 0.09	*p* = 0.58	1.79	0.12
	Model2:	F (1, 18) = 6.28	F (1, 18) = 4.49	F (1, 18) = 0.05	F(3, 18) =	
	Heterosp. Male groups	***p* = 0.02**	***p* = 0.05**	*p* = 0.82	3.61	**0.03**

Appendix Table 2.5 Regression models predict gene (*neuroserpin*, *egr-1* and *early B*) residuals with TT (total transit) and social exposure (Model 1: C_fC_F, C_mC_M, C_mH_{cr}, and C_mH_{ct}; Model 2: C_mH_{cr} and C_mH_{ct}). (**a**)regression coefficient (intercept or slopes of the linear relationship between TT and gene residuals) and *p* values for each group-specific predictor, along with the overall R^2 and R^2-adjusted. (**b**) Analyses of Variance of the six models shown in (**a**). *neuroserpin* exhibited overall significance for model 1 and model 2 while *early B* was significant by model 2 only.

(a)

			Estimate (*p*-value)				Overall	
Response			**C_fC_F**	**C_mC_M**	**C_mH_{cr}**	**C_mH_{ct}**	R^2	R^2_{adj}
neuroserpin residual	Model 1:	Intercept	-0.19 (0.58)	0.24 (0.60)	-0.17 (0.66)	-0.22 (0.67)		
	All social groups	TT coefficient (slope)	0.01 (0.17)	-0.01 (0.33)	-0.01 (0.33)	0.01 (0.49)	0.48	0.18
	Model 2:	Intercept			-0.36 (**0.04**)	-0.05 (0.91)		
	Heterosp. Male groups	TT coefficient (slope)			0.003 (0.43)	0.02 (0.10)	0.39	0.29
egr-1 residual	Model 1:	Intercept	-0.06 (0.90)	0.08 (0.89)	-0.28 (0.56)	-0.25 (0.70)		
	All social groups	TT coefficient (slope)	0.007 (0.59)	-0.009 (0.63)	-0.003 (0.83)	0.02 (0.33)	0.23	0.08
	Model 2:	Intercept			-0.34 (0.12)	0.03 (0.96)		
	Heterosp. Male groups	TT coefficient (slope)			0.004 (0.46)	0.02 (0.18)	0.34	0.23
early B residual	Model 1:	intercept	0.02 (0.96)	0.15 (0.74)	-0.34 (0.35)	-0.20 (0.69)		
	All social groups	TT coefficient (slope)	0.005 (0.6)	-0.01(0.43)	-0.001 (0.91)	0.01 (0.43)	0.25	0.1
	Model 2:	Intercept			-0.33 (**0.05**)	0.14 (0.72)		
	Heterosp. Male groups	TT coefficient (slope)			0.004 (0.32)	0.01 (0.29)	0.34	0.23

(b)

		Factors			overall	
Response		Groups	Total transit	Groups x Total transit	F	*p*-value
neuroserpin residual	Model1:	F (3, 35) = 3.03	F (1, 35) = 3.24	F (3, 35) = 1.39	F (7, 25) =	
	All social groups	***p* = 0.05**	*p* = 0.08	*p* = 0.26	2.36	**0.04**
	Model2:	F (1, 18) = 5.77	F (1, 18) = 4.63	F (1, 18) = 0.07	F(3, 18) =	
	Heterosp. Male groups	***p* = 0.02**	*p* = 0.16	*p* = 0.10	3.81	**0.03**
egr-1 residual	Model1:	F (3, 35) = 2.09	F (1, 35) = 1.74	F (3, 35) = 0.81	F (7, 25) =	
	All social groups	*p* = 0.12	*p* = 0.20	*p* = 0.50	1.49	0.2
	Model2:	F (1, 18) = 5.50	F (1, 18) = 1.67	F (1, 18) = 1.96	F(3, 18) =	
	Heterosp. Male groups	***p* = 0.03**	*p* = 0.21	*p* = 0.18	3.04	0.06
early B residual	Model1:	F (3, 35) = 2.47	F (1, 35) = 1.77	F (3, 35) = 0.78	F (7, 25) =	
	All social groups	*p* = 0.08	*p* = 0.19	*p* = 0.52	1.65	0.16
	Model2:	F (1, 18) = 5.95	F (1, 18) = 2.16	F (1, 18) = 1.17	F(3, 18) =	
	Heterosp. Male groups	***p* = 0.03**	*p* = 0.16	*p* = 0.29	3.09	**0.05**

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