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Sex Selectivity of Mouse Ultrasonic Songs

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Abstract

In many species, reproduction requires detecting, recognizing, and courting a potential mate. Progress through these stages is guided by cues involving a wide range of sensory systems. Here we explore the tasks of detection, recognition, and response in terms of the ultrasonic songs of male mice presented with odor cues contained in urine. We find that the quantity of singing, more so than specific features of the songs, varies depending upon the odor cue. For experienced male mice, responses to female odor cues depend only on the concentration of female cues and are independent of the presence of male cues. However, for naive mice, male cues appear to be synergistic for the response to female cues. We therefore find no direct behavioral evidence for a role of opponent neural processing, such as lateral inhibition, in distinguishing sex by olfactory cues. However, modeling demonstrates that lateral inhibition could be one possible mechanism to account for the switch from synergy to independence.

Key words: lateral inhibition, olfaction, opponency, psychophysics, sex recognition

Introduction

Previous work has demonstrated that common laboratory mice, of the species *Mus musculus*, make ultrasounds (Sales 1972) and that males emit these ultrasounds in the presence of females or when they detect olfactory cues (pheromones) of females (Nyby et al. 1977, 1979). It has been proposed that these ultrasounds have a role in courtship (Nyby et al. 1977); this interpretation is supported by the circumstances of their emission and by the observation that females are attracted to vocalizing males (Pomerantz et al. 1983). However, definitive proof that these vocalizations contribute to sexual behaviors in the mouse, as has been demonstrated in rats (McIntosh et al. 1978), is lacking.

Recently, we reported (Holy and Guo 2005) that these ultrasonic vocalizations have the characteristics of song: they consist of several distinct syllable types arranged in nonrandom temporal sequences and with reproducible individuality in song among different, genetically identical males. We have since become aware of a much older literature reporting that rare individuals, for reasons not fully elucidated, sing at audible frequencies (Farr 1857; Lockwood 1871; Dice 1932) and thus directly appreciable to human hears. Indeed, in 1877, the son of Sidebotham (1877) speculated that singing might be common in *M. musculus* but occurring typically at ultrasonic frequencies. Our previous study (Holy and Guo 2005) showed this to indeed be true and documented the behavior with a thoroughness that would have been impossible in earlier times.

The purpose of the present study is to examine how mouse song depends on stimulus cues. This overall goal can be broken into 2 components: first, do male mice have different songs triggered by different stimulus cues? For example, do males use one song in the presence of female cues (presumably for courtship) and another to male cues (perhaps for territorial or aggressive display)? Among birds, there are many examples of context dependency in song type (Nelson and Croner 1991, Table 3), although it is also common for the same song type to serve both aggressive and courtship functions, depending on the receiver rather than the singer (Lein 1972; Smith and Reid 1979).

Second, can the songs be used to obtain a behavioral report of the process of the detection and recognition of relevant stimulus cues? In particular, are the pheromonal cues of males antagonistic for the recognition of female cues or are these independent communication channels? Such antagonism, or "opponency," is a mechanism used, for example, in the retina (Hering 1920; Hurvich and Jameson 1957) to distinguish changes in stimulus identity (color) from stimulus intensity (brightness), an ambiguity that arises from the relatively broad spectral tuning of cone photoreceptors. In the olfactory system, both the tuning of receptors (reviewed in Firestein

2001) and synaptic organization of the olfactory bulb (Shepherd 1998) are, at least in some senses, reminiscent of the retina. Indeed, a phenomenon of "lateral inhibition," perhaps sharpening odor representations, has been described (Yokoi et al. 1995; Mori et al. 1999; Urban 2002). However, it has also been pointed out that the large number of odorant receptor types enables novel forms of computation (Hopfield 1999) and indeed that such sharpening might not actually improve discriminability (Laurent 1999). Thus, although it seems clear that opponent or inhibitory mechanisms exist within the olfactory bulb, their functional role in the perception of odors is uncertain.

In the context of male mouse ultrasonic songs, previous literature addresses some of these questions, at least to a limited extent. Nyby et al. (1979) found that ultrasonic vocalizations were more abundant when triggered by female mouse urine than by male mouse urine. However, due to the choice of recording methods, no information exists about whether the acoustic details of these vocalizations differ. Similarly, the same study observed that a 50–50 mixture of male and female mouse urine is as effective as female mouse urine in evoking vocalizations from experienced animals. This does not, however, demonstrate that perception of sex depends solely on cues in female mouse urine: abundant examples in other sensory systems—and particularly those demonstrating opponency—show that the perception of mixtures of 2 different cues is often dominated by one or the other cue depending on the ratio of the 2 components. For example, in color vision, we do not perceive "reddish–greenish": a color is categorized as either red or green depending upon the ratio of the 2 components (Hering 1920). Similarly, native speakers of English perceive mixtures of /r/ and /l/ sounds as one or the other depending upon the ratio of the 2 components (Iverson and Kuhl 1996).

For these reasons, we undertook a systematic study to better determine the relationship between songs and olfactory stimulus cues.

Materials and methods

Signal acquisition and testing environment

Sounds were acquired as described (Holy and Guo 2005). Odors were presented on cotton swabs (20 μl) mounted in a custom detector. Approach to the odorous face of the cotton swab breaks the beam of an infrared (935 nm) LED; the Schmitt-trigger detector (Honeywell, Morristown, NJ) outputs a transistor-transistor logic (TTL) signal, which was captured at 1 kHz by the same acquisition card and software to insure that sound and detector recordings were synchronized.

Urine collection and preparation

Urine was collected from BALB/c males and females, housed in wire-bottom cages suspended above trays of liquid nitrogen. Frozen urine was collected daily and stored at −86 °C. After 2 weeks, urine was thawed, pooled by sex, and clarified by mild centrifugation (3000 × g for 30 s) before being stored again at −86 °C. Urine and urine mixtures were diluted with water to achieve their final concentrations.

Experimental design

Mice, housing conditions, and the trial sequence are as described (Holy and Guo 2005). All procedures were approved by the Animal Studies Committee of Washington University in St Louis.

Over the course of 7 weeks, each male completed the following sequence twice: weeks 1 and 2, mixture stimuli and week 3, unambiguous stimuli (0.316× male and 0.316× female mouse urine). In the first cycle, mice were socially naive. After the first 3-week cycle, males were given two 3-min social experiences per day for 4 days, one to a BALB/c female and one to a BALB/c castrated male painted with 50-μl 0.316× intact BALB/c male mouse urine (Stowers et al. 2002). The order of female/male social experiences was balanced across days. The males then repeated the 3-week stimulus sequence. Mixture stimuli were selected in a balanced random fashion to insure that each stimulus was presented 20 times to 20 different mice in each cycle and that no mouse was exposed twice within a cycle to the same mixture.

Data analysis

Ultrasounds were analyzed as described (Holy and Guo 2005). Periods of swab investigation were indicated by the TTL signal from the optical detector. To exclude false triggers, for example, from detector movement and changes in lighting when inserting the detector into the chamber, we discarded bouts of investigation lasting less than 10 ms.

For the analysis of syllable prevalence (Figure 3), female trials were matched to male trials by selecting random sets of trials without replacement and performing a paired *t*-test on the trials, sorted by total number of chirps. Only sets with $P > 0.2$ were considered matching, and a total of 1000 such sets were generated.

Two-way nonparametric analysis of variance (ANOVA) employed Friedman's test, used once to check for male effects and again for female effects. For experienced mice, a single trial was missing (see Figure 5) due to a failure of the mouse to approach the swab. Because Friedman's test requires equal numbers of trials for each specific combination of male and female mouse urine concentrations, 19 of the 19 or 20 available trials in each combination were selected randomly without replacement for ANOVA analysis. The reported *P* values are averages over 100 realizations of this random selection. Without exception, individual realizations were consistent with the reported *P* values in terms of the statistical significance of the results.

Modeling

Steady-state firing rate models were used for olfactory circuits. We assumed that the activation of a receptor neuron arose from a single receptor-binding site, which might be activated by either or both of the 2 stimuli in a mixture. The normalized firing rate r of a receptor neuron was therefore

$$r = \frac{c_1/K_1 + c_2/K_2}{1 + c_1/K_1 + c_2/K_2}, \quad (1)$$

where c_1 and c_2 are the concentrations of the 2 components and K_1 and K_2 are the affinities (EC_{50}) for each of the 2 components. Model mitral cells responded to excitatory input current I (where I is a vector of inputs, one per mitral cell) by firing at rates f determined by

$$(1+A)f = I/q_{th} - 1/(2\tau), \quad (2)$$

where τ is the membrane time constant, $q_{th} = CV_{th}$ is the charge needed to bring a membrane with capacitance C to a threshold voltage V_{th}, and A is a matrix describing the effective inhibition from lateral connections in the bulb. The derivation of this equation will be described elsewhere (Neeman O, Holy TE, in preparation). Equation (2) was solved in a least-squares sense, constraining the mitral cell firing rates to be nonnegative. Additional parameters of the model were factors of proportionality α converting sensory neuron activation, equation (1), to input current I in the mitral cells ($I_i = \alpha_i r_i$) and a factor converting mitral cell firing rate to behavioral output, the chirp rate (chirp rate = βf_1).

Results

Male mice of the B6D2F1 strain were presented with sex-specific odors applied on cotton swabs. We used dilute urine of either sex (BALB/c, so that urine stimuli of both sexes were unfamiliar to the tested male) and mixtures of urine from both sexes. As described previously (Holy and Guo 2005), we recorded all sounds in the chamber with a microphone sensitive to frequencies from 20 Hz to 100 kHz and higher. We also simultaneously detected the timing of episodes in which the male explored the cotton swab via the breaking of an infrared beam positioned immediately in front of the swab. An example of the vocal responses to both male and female mouse urine is shown in Figure 1.

Responses to unambiguous odors: socially experienced males

We examined the olfactory triggers for these songs by presenting 45 socially experienced subject males (B6D2F1; social experience denotes a history of brief adulthood contacts

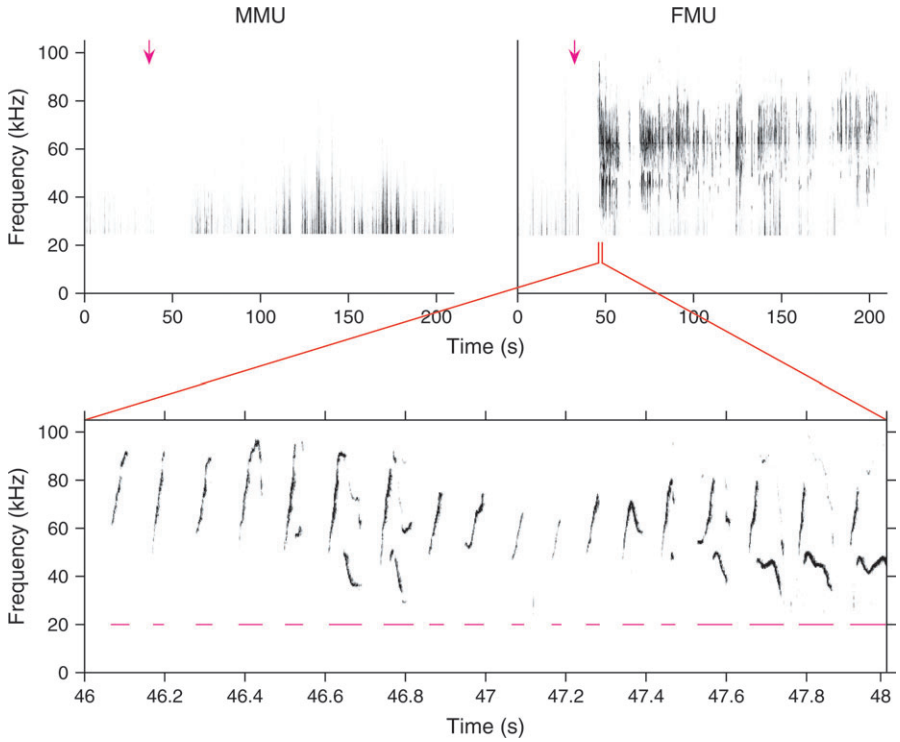

Figure 1 Male mice vocalize in the ultrasound in response to urinary cues. A cotton swab containing either male mouse urine (MMU, top left) or female mouse urine (FMU, top right) was introduced at approximately 30 s into a 210-s trial. Arrows indicate the time of first contact with the cotton swab, as signaled by an optical detector. Recorded acoustical power is represented as a function of time and frequency, with shading increasing with power. Power below 25 kHz was truncated. Bottom, an expansion of a 2-s period showing vocalizations in greater detail. Individual chirps, as identified by an automated algorithm, are spanned by magenta lines below.

with both males and females, see Materials and methods) with odors on cotton swabs. We employed 3 stimuli: a cotton swab with male mouse urine (M), a blank cotton swab (0), and a cotton swab with female mouse urine (F). To permit direct comparison with mixture experiments described below, urine (BALB/c) was diluted with water to a concentration 0.316× relative to full strength.

As a first characterization of the vocal response, we quantified the "chirp rate," using the time of onset of each syllable as measured by an automated algorithm (Figure 1 and [Holy and Guo 2005]). Chirp rates for all 45 males to the 3 stimuli are shown in Figure 2. Most notably, the chirp rate triggered by female cues was far larger than the chirp rate to male cues or a blank swab. When averaged across mice, the chirp rates triggered by female cues reached a peak in 25 s after initial exploration of the swab and maintained at least half the peak rate over the next 100 s (Figure 2A). The chirp rates, averaged over all mice and time, showed highly significant ($P < 10^{-5}$, Wilcoxon rank sum test) differences between trials with female mouse urine and the other stimuli (Figure 2B). When averaged over time, the chirp rate varied widely over mice (Figure 2C), but it is apparent that most males sang in response to female cues (35/45 at a rate higher than 0.25 chirps/s following initial contact with the swab), whereas only a small minority of mice sang to male cues or blank swab. Of the small subset that sang to male cues (6/45 at a chirp rate higher than 0.25 chirps/s following initial contact with the swab), the majority (4/6) were singing even prior to the introduction of the cotton swab (Figure 2A). Five out of 6 of these mice had sung on trials conducted previously (see Responses to mixtures: experienced males, Responses by naive mice, and Materials and methods) and may have learned to associate the testing environment with female cues (Sipos et al. 1992). The robustness of the response selectivity is highlighted by the fact that a simple threshold, set anywhere within 0.1–0.9 chirps/s, distinguished female trials from other trials with a success rate higher than 80% given 50–50 odds. Therefore, the vocalization responses in a single trial were quite informative about the presence or absence of female cues.

We wondered whether the (relatively rare) bouts of song when male cues were presented might differ from the songs initiated by female cues. To examine this possibility, we first tested whether the features used to characterize syllables previously (Holy and Guo 2005) for songs triggered by female cues were also present for songs in trials with male cues. Apart from a substantial difference in the number of chirps, Figure 3 shows that the same pitch-jump features are present in the songs to both types of cues. However, the abundance of different syllable types (Holy and Guo 2005) might depend on the olfactory stimulus. To test this, we counted the numbers of each common syllable type uttered after the initial contact with the swab, including only trials with at least 10 chirps after initial contact with the swab (39/45 for female trials and 11/45 for male trials). For some syllable

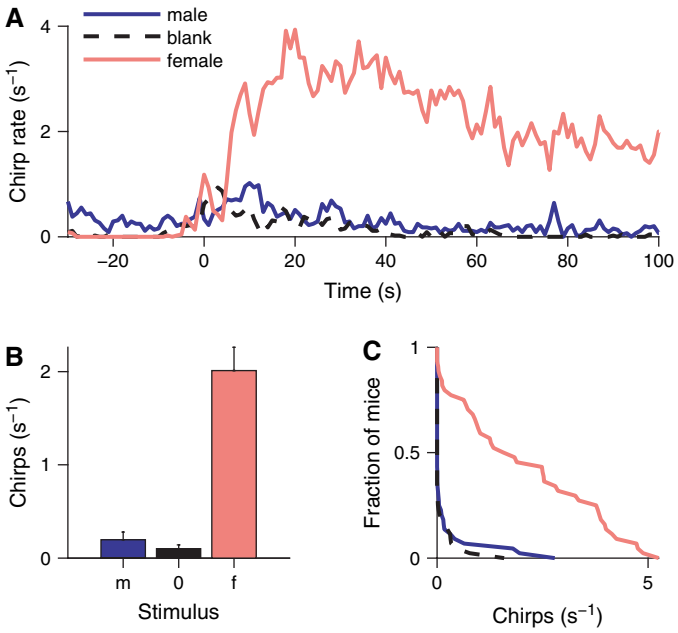

Figure 2 Vocal responses to unambiguous stimuli, as measured by the chirp rate. **(A)** Chirp rate as a function of time for male, blank, and female odor cues. Urine stimuli were diluted to a concentration of 0.316. $t = 0$ corresponds to the first contact with the cotton swab. Mice were tested in the following order: male, blank, and female. Note the precontact chirp rate reduces considerably after the first trial. **(B)** Postinvestigation chirp rates averaged across time and mice. Error bars are standard error of mean **(C)** Cumulative distribution across 45 mice of average chirp rate from the moment of first investigation to the end of the 210-s trial.

types, the differences in the population means were statistically significant (Figure 3C, dark error bars). However, much of this difference can be attributed to the fact that the percentage of the more "complex" syllables was quite strongly correlated (e.g., $r = 0.79$ in the case of "du") with the total number of syllables; this suggests that the percentage of such syllables might be another measure of the "enthusiasm" of the vocal response. When this correlation was controlled by selecting subsets of female trials that were matched in total number of syllables to the male trials (see Materials and methods), the mean abundances of all but 2 syllable types (d and hdu) were statistically indistinguishable between stimuli (Figure 3D). This might be taken to indicate that "d" and "hdu" syllables were uttered in a stimulus-specific fashion. However, 2 caveats should be noted: first, individual males had characteristic patterns of syllable usage (Holy and Guo 2005), and for this data set, it was not possible to match both male identity and total number of chirps; consequently, the differences might be attributable to the singer rather than the stimulus. Second, the difference in the mean across trials, although statistically significant, was substantially smaller than the standard deviation across trials (Figure 3C, gray error bars). Accordingly, the proportion of each syllable type was unreliable as a predictor of stimulus identity: in the most favorable case (hdu), an optimally chosen threshold performed better than chance by only 5%. This is

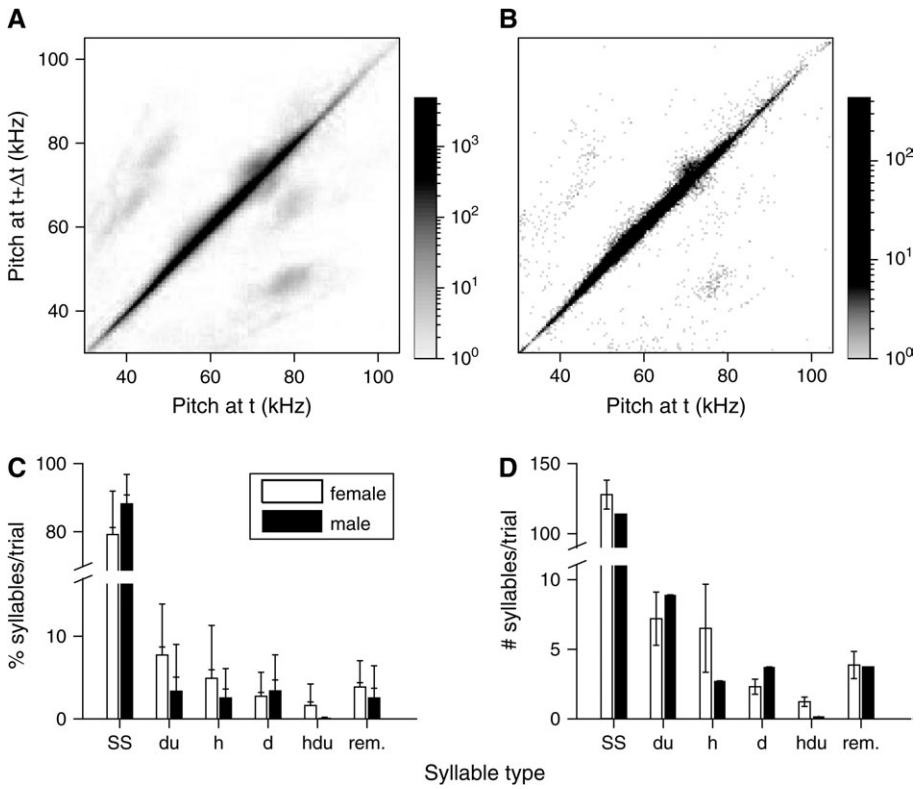

Figure 3 Song features in response to unambiguous stimuli. **(A)** Pitch changes (Holy and Guo 2005) in response to female stimuli, cumulatively across all 45 trials. Pitch is plotted at one time point versus the next time bin, 1.02 ms later. Only syllables occurring after initial contact with the swab are included. Total number of observations in each bin (256 by 256) is indicated by the color scale to the right. **(B)** The identical analysis performed for the 45 male cue trials. **(C)** Percentage of different syllable types (Holy and Guo 2005) averaged across trials containing at least 10 postinvestigatory chirps (female: 39 trials; male: 11 trials). Short error bars show the standard error of mean (SEM); tall error bars the standard deviation. **(D)** Number of different syllable types in randomly generated sets of 11 female trials matched, in terms of total number of chirps, to the male trials. Error bars represent the standard deviation of the average across trials (i.e., SEM) across the 1000 randomly generated trial sets.

in distinct contrast with the ease with which singer identity can be deduced from single trials on the basis of syllable type usage (Holy and Guo 2005).

We also examined the temporal structure of the songs, in terms of the probability of switching between syllable types. As with our previous analysis (Holy and Guo 2005), we divided the syllables into only 2 types, those with (1) and without (0) low jumps and then calculated the probability $P_{1\rightarrow 0}$ that a syllable of type "1" is followed by a syllable of type "0." No significant difference in the transition probabilities between trials with male and female cues could be found ($P = 0.29$, rank sum test).

Overall, therefore, we find little evidence for differences in the detailed features of the songs conditional on male versus female cues. The principal difference appears to be the total amount of singing, which was much higher for female cues than for male cues (Figure 2).

In addition to recording their vocal output, we also recorded the timing of episodes in which the male investigated the cotton swab, as signaled by the optical detector (see Materials and methods). Because the detector indicates proximity rather than any specific information about the mouse's behavior, these periods included bouts of chewing, tugging, and climbing the detector as well as periods of apparent olfactory exploration. We noted that these "distractor" behaviors, when present, tended to occur later in the trial. When averaged across mice, the likelihood of investigating the swab appeared to show sizable differences between trials with female cues and other trials in the first 25 s after initial contact (Figure 4A). We therefore computed the fraction of this initial period spent exploring the swab. This parameter too varied widely across mice, yet showed significant ($P < 10^{-5}$) odor-specific differences, albeit with much smaller ratio than the chirp rate (Figure 4B,C). Despite the wide variation, the fraction of time spent sniffing was also a quite reliable indicator of the stimulus identity, as thresholding this parameter permitted correct identification more than 80% of the time when female and nonfemale trials were chosen with equal likelihood. Later in the trial, there was a weak tendency to avoid cotton swabs with male cues (Figure 4A); the fraction of time spent investigating the swab after the initial 25-s period allowed discrimination between male and nonmale stimuli with a peak accuracy of approximately 65%.

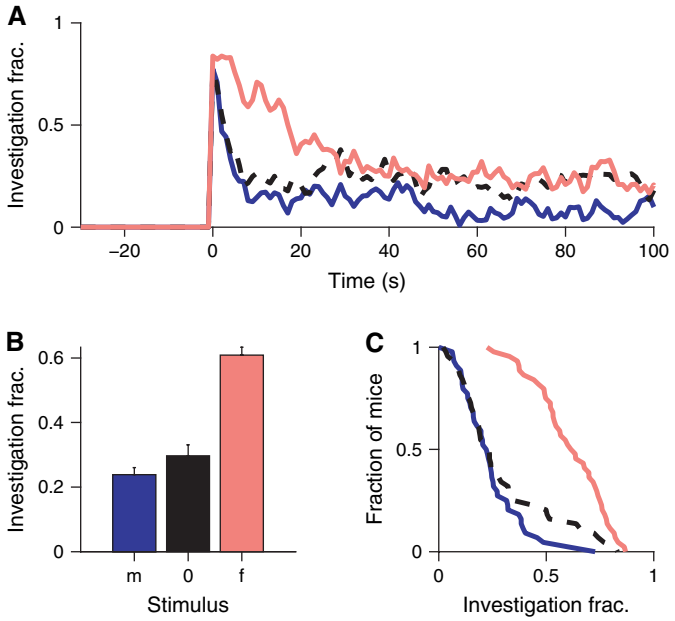

Figure 4 Investigatory responses to stimuli. **(A)** Fraction of time spent investigating the swab, as a function of time through the trial. Color scheme and timing are as in Figure 2. Curves do not reach 1.0 at $t = 0$ because the initial investigation does not always last the full bin width, 1 s. **(B)** Fraction of time spent investigating the swab, averaged across time and mice. **(C)** Cumulative distribution across mice of the fraction of time spent investigating the swab during the initial 25 s after first investigation.

Responses to mixtures: experienced males

To learn more about the psychophysics of olfactory sex recognition, we also presented these 45 socially experienced B6D2F1 males with mixtures of male and female BALB/c urine stimuli at a range of concentrations. In each mixture, the final concentration of each sex's urine was one of 0.0316, 0.1, and 0.316 relative to full strength; all 9 possible mixtures were presented a total of 20 times in random order to 20 different males with no male receiving the same stimulus twice. In an attempt to minimize a learned association of the testing environment with female cues (Sipos et al. 1992), these mixture experiments were completed prior to the experiments with unambiguous stimuli described above (see Materials and methods).

We computed the chirp rate, averaged across mice and time (between initial exploration of the swab and the trial's end), for each of the 9 stimuli. Males responded more strongly with increasing concentration of female cues (Figure 5A). To determine whether this reflected an increase in the typical chirp rate, an increase in the number of responsive mice, or both, we divided trials into "responsive" and "nonresponsive" depending (Figure 2C) on whether the postinvestigation chirp rate exceeded 0.25 chirps/s. As can be seen in Figure 5B, the number of positive trials agrees fairly well with the mean chirp rates, suggesting that much of the difference in mean chirp rates arises from the number of responsive mice.

A 2-way nonparametric ANOVA revealed a significant ($P < 10^{-4}$) effect on chirp rate of the concentration of female mouse urine but no evidence ($P = 0.54$) for a dependence on the concentration of male urine. This result is corroborated by averaging responses across the different concentrations of male and female mouse urine (Figure 5C). Further insight is obtained by analyzing the fraction of time spent investigating the swab during the first 25 s after initial exploration (Figure 5D). As with the unambiguous stimuli (Figure 4), males spent more time exploring swabs with increasing concentration of female cues. A 2-way nonparametric ANOVA demonstrates a significant ($P < 10^{-5}$) role for female cues but not ($P = 0.87$) for male cues. This result is also reflected in the chirp rates averaged over all trials containing a particular concentration of cues from either sex (Figure 5E).

Although both chirp rates and exploration times appear to be independent of male cues, the mean chirp rate to the 3 mixture stimuli containing 0.316× female mouse urine (Figure 5C) was 0.69 ± 0.13 chirps/s, substantially below the mean chirp rate (2.01 ± 0.25/s) to unambiguous female mouse urine at the same concentration (Figure 2B). This could indicate a role for male cues not revealed by Figure 5. Alternatively, we noted that the responsiveness to these cues was not stationary in time: over the course of 3 weeks, the mean chirp rate to high (0.316×) female-content stimuli approximately doubled each week (Figure 5F). Because the mixtures were used in the first 2 weeks, and pure stimuli were employed in week 3, it seems possible that this discrepancy arises from the order in which stimuli were presented.

Responses by naive mice

The males used in these studies had recent, brief exposure to other mice of both sexes (see Materials and methods). To determine the role of experience, we had previously conducted the same set of experiments (2 weeks with mixtures of male and female mouse urine and a third week of unambiguous stimuli) on these mice before social experience. In partial contrast with experienced mice, we found that chirping responses in naive animals were highly dependent upon the animal's history of stimulus exposure: mice who responded in an early trial tended to respond indiscriminately to later stimuli. For example, in trials with unambiguous stimuli (presented during week 3) using naive mice, the distributions of chirp rates to male and female mouse urine stimuli were not different (Kolmogorov–Smirnov test, $P \approx 0.3$). For the mixture trials, a one-way ANOVA analysis revealed a significant relationship between stimulus identity and chirp rate on the males' very first trial ($P < 0.002$) but not for any of the 3 successive mixture trials ($0.1 < P < 0.9$). More directly, a mouse's chirp rate on both the male and female trials was significantly correlated (male, $r_s = 0.49$; female, $r_s = 0.52$; $P < 0.001$, Spearman's rank-order correlation) with its chirp rate on the very first trial. Indeed, if chirp

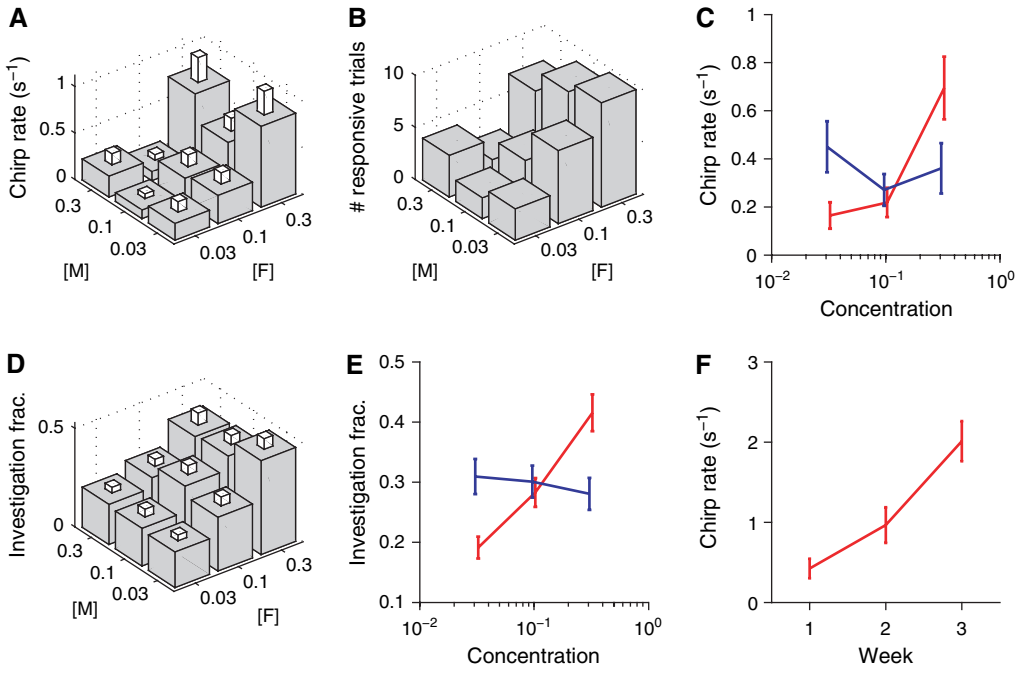

Figure 5 Responses to mixture stimuli by experienced mice. **(A)** Chirp rate, averaged across time and mice, following initial investigation of cotton swabs with 20 μl of mixed urine stimuli from male and female mice. "Error boxes" are standard error of mean. Each represents an average across 20 trials, except for one stimulus (0.0316 male, 0.0316 female) with 19 trials, as in one trial the mouse failed to approach the swab. **(B)** Number of positive responses to each mixture stimulus; positive trials were those in which the average postinvestigation chirp rate was greater than 0.25 chirps/s. **(C)** Marginal chirp rates. For the curve representing female cues (red), each data point represents the average of responses to all 3 stimuli with a given concentration of female mouse urine; the curve for male cues (blue) is its complement, averaging across the different concentrations of female mouse urine. To prevent the error bars from overlapping, curves are slightly shifted apart horizontally. **(D)** Fraction of time spent investigating the swab during the first 25 s after initial contact. **(E)** Marginal investigation times, similar to panel (C). **(F)** Mean chirp rate to all stimuli containing 0.316× female mouse urine, as a function of time throughout the experiment. Mixture stimuli were used in weeks 1 and 2 and unambiguous stimuli in week 3.

rates above a threshold r_c are scored as positive, then for any r_c between 0 and 2 chirps/s, more than half of the naive males responding to any stimulus did so on their first trial. We conclude that, for naive males, the chirp rate is not a reliable indicator of stimulus identity after their first responsive trial. However, chirp rates in the first trial were significantly ($P < 0.002$) related to the stimulus, so we analyzed the responses in this trial to determine the nature of this relationship. It is noteworthy that these subjects were naive with respect to both social experience and the trial experience. Across the 45 males, there were 5 presentations of each of the 9 mixture stimuli.

In contrast to the results with socially experienced mice (Figure 5A), mean chirp rates depended on the concentration of male as well as female cues (Figure 6A). Whereas female cues appear to dominate, male cues act synergistically, not antagonistically, to female cues. A 2-way nonparametric ANOVA revealed a significant role for both female ($P = 3 \times 10^{-4}$) and male ($P = 0.004$) cues. As with experienced animals (Figure 5B), we also counted the number of trials with chirp rates above 0.25 chirps/s (Figure 6B) and found that these differences in chirp rates are mirrored, and perhaps explained, by the differences in the fraction of responsive males.

Again in contrast to the socially experienced case, the fraction of time spent investigating the swab, during the first 25 s after initial contact, was largely independent of stimulus identity or concentration (Figure 6D). Therefore, the differences in chirp rates do not arise from a difference in the total stimulus exposure.

We also analyzed specific song features triggered by the stimulus mixtures. To ensure adequate statistics, here we classify syllables into only 2 types, 1 containing low jumps and 0 without low jumps (Holy and Guo 2005). When the fraction P_1 of type 1 is plotted, no consistent stimulus-dependent pattern emerges for either experienced or naive mice (Figure 7A,C). Similarly, the transition probabilities $P_{1\rightarrow 0}$ also show no obvious pattern of stimulus dependency (Figure 7B,D). Therefore, consistent with the results in experienced males (Figure 3), we find little evidence for differences in the detailed features of songs triggered by different mixture stimuli.

Does a lack of opponency exclude a role for lateral inhibition in olfactory sex recognition?

Neither naive nor experienced mice showed direct behavioral evidence for opponent processing: in no case was male mouse

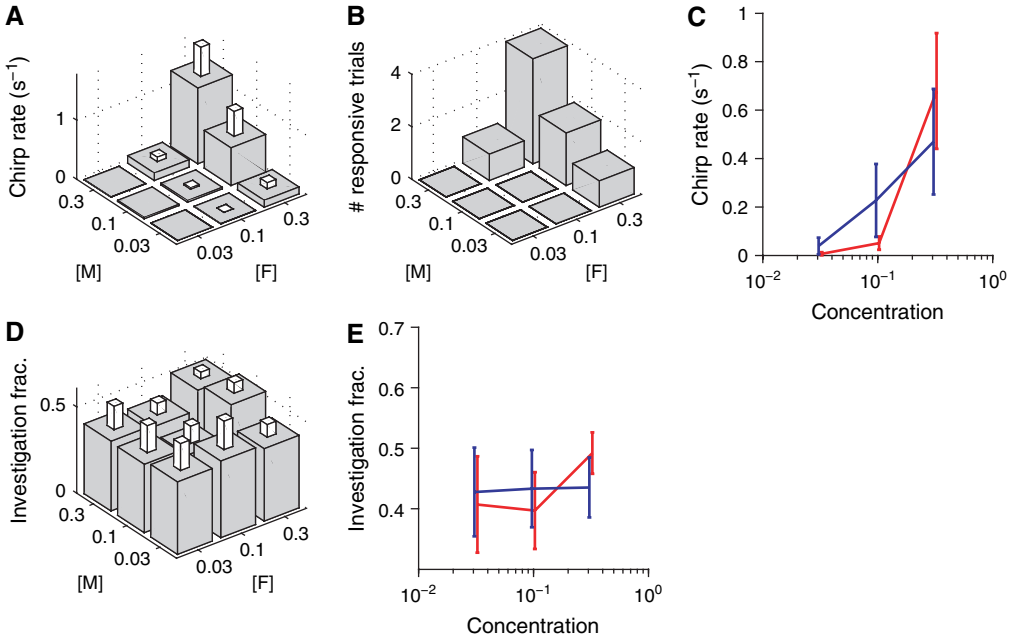

Figure 6 Responses to mixture stimuli by naive mice. Panels are as in Figure 5. Five trials of each of the 9 stimuli were used.

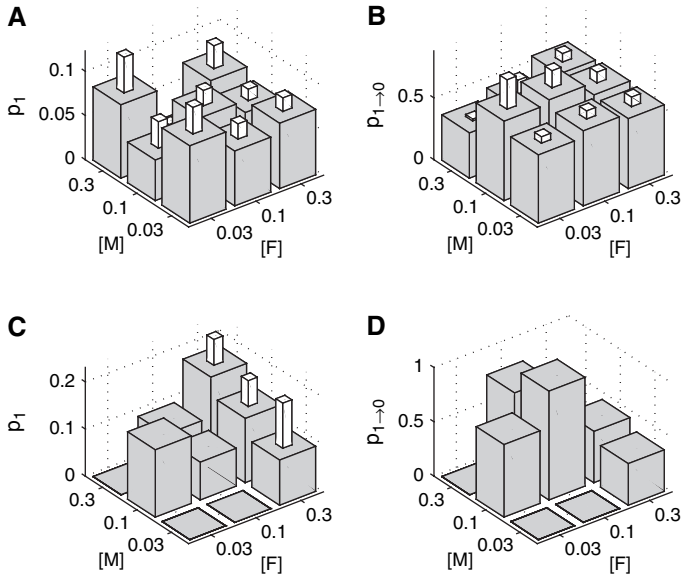

Figure 7 Syllable type usage during mixture trials. **(A)** Fraction of syllables containing low jumps (LJ$^+$, i.e., "d" or "u" jumps, [Holy and Guo 2005]) for trials with socially experienced mice, plotted as a function of mixture composition. Each bin contains an average across 20 trials, except as described in Figure 5. **(B)** Transition probability (Holy and Guo 2005) to switch from LJ$^+$ syllables to LJ$^-$ syllables for experienced mice. **(C)** Syllable usage as in panel (A) for naive mice. Each bin is the average across 5 trials. **(D)** Transition probability as in panel (B) for naive mice.

urine clearly inhibiting the response to female mouse urine. This could be taken as evidence against a functional role for inhibitory neural processing in this particular task. However, it might be argued that inhibition could account for the shift from synergy, in the case of naive animals, to neutrality, in the case of experienced animals. To test this possibility, we examined a model of lateral inhibitory processing in the olfactory bulb (see Materials and methods). We fit the chirp rate, averaged across animals and trials, to the firing rate of a single mitral cell. A simple circuit with a single receptor neuron type with less than 2-fold selectivity for female mouse urine was able to reproduce the qualitative features of the response by naive animals (Figure 8A,C). The response by experienced animals was qualitatively reproduced by adding a second, nonselective receptor type and coupling it through an inhibitory network to the output of the chosen mitral cell (Figure 8B,D). Thus, we conclude that we cannot rule out a role for lateral inhibition in this task.

Discussion

This study examined how the songs of male mice, triggered by olfactory cues, differed depending on stimulus. In some species, the presence of females elicits vocal responses that differ from those used in agonistic encounters with males (Nelson and Croner 1991); however, it is also rather common for species with song repertoires to be indiscriminate in their song patterns (Lein 1972). An interesting example is seen in red-winged blackbirds: males sing 5–7 different song types but do not display context dependence in their song type usage (Smith and Reid 1979), whereas (unusually among birds) female red-winged blackbirds also sing, and their 2 song types function separately in pair-bond maintenance and territorial defense (Beletsky 1983). In the odor-triggered songs of B6D2F1 laboratory mice, the main difference appears to be quantity, rather than type, of vocal response: we

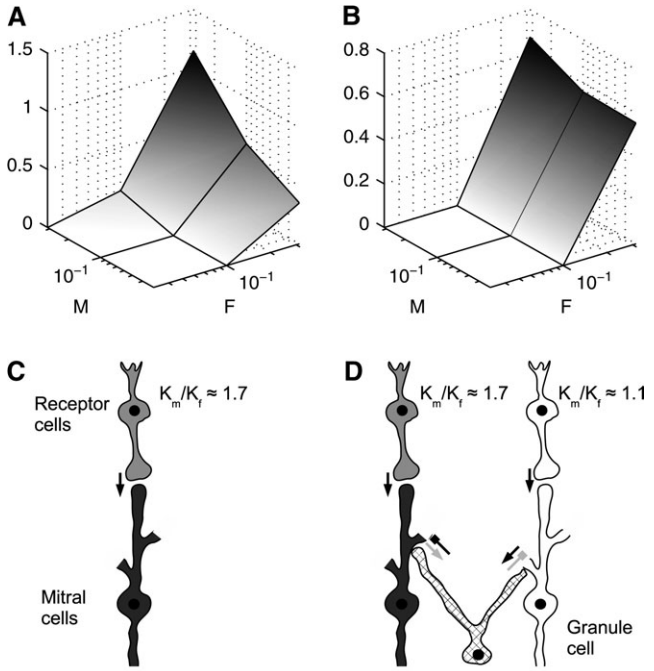

Figure 8 Olfactory lateral inhibition could provide one mechanism (among other possibilities) for the switch from synergy to independence. Output **(A)** of a model **(C)** involving a single, modestly female-selective receptor type and its corresponding mitral cell. The specificity of the receptor is characterized by the ratio $K_m/K_f \approx 1.7$ of binding affinities for male (K_m) and female (K_f) urine stimuli, respectively. Behavioral response (chirp rate) is postulated to be proportional to mitral cell firing rate and shows synergy because of the modest selectivity of the receptor type. Output **(B)** of a model **(D)** in which a second, largely nonspecific ($K_m/K_f \approx 1.1$) inhibitory pathway suppresses the synergistic effects of male cues. In the diagram (D), strong synaptic pathways are indicated by dark arrows, whereas weak (or negligible) pathways are indicated in gray. Output is proportional to the firing rate of the mitral cell on the left.

find that socially experienced mice sing reliably to female but not male odors (Figure 2). This result is consistent with a number of previous studies (Nyby et al. 1979; Wysocki et al. 1982; Sipos et al. 1992) that have shown that the mean rate of vocalizing is much higher to female rather than male mouse urine and is consistent with a role for these songs in courtship (Pomerantz et al. 1983). We have found little evidence for the presence of specific syllable types, or changes in the distribution of syllable usage, in the uncommon cases when males sang when presented with male mouse urine. Of course, it is possible that longer trials (or more directly antagonistic circumstances) would improve the possibility of observing very fine differences in the qualitative features of these songs.

We find no direct evidence for opponency in the behavioral sex recognition task, whether one considers the chirp rate or, for experienced animals, the time spent investigating the swab. One interpretation of this result is that processes such as lateral inhibition (Yokoi et al. 1995) play no role in the recognition of sex, at least as measured by singing. However, this interpretation is complicated by the switch from synergy (in naive mice) to independence (in experienced mice). Indeed, modeling demonstrates (Figure 8) that such a switch is explainable in terms of the development of lateral inhibition. The fact that olfactory learning is known to occur in the accessory olfactory bulb (Brennan and Keverne 1997) makes such a mechanism plausible. Of course, these data might also be explained by other changes, such as a switch in the choice of receptor type driving the behavior, or alterations in the degree of synchrony among mitral cells (Laurent et al. 2001; Brody and Hopfield 2003). Indeed, these changes do not have to be confined to the olfactory centers of the brain: one alternative is that experience leaves the olfactory percept unaltered but instead increases the degree to which perceived sex matters in deciding to initiate a behavioral output.

In the visual system, opponency permits fine color discrimination with broadly tuned receptors. How are the receptors for sex recognition tuned? For this task, detection and recognition are likely carried out in the accessory olfactory (also known as vomeronasal) system: both surgical ablations (Wysocki et al. 1982) and genetic knockout (Stowers et al. 2002) of the responses of sensory neurons in the vomeronasal organ (VNO) result in profound alterations in vocal responses to sex-specific chemosensory cues. Similarly, the excess time spent exploring cotton swabs containing female mouse urine was previously shown to be VNO dependent (Pankevich et al. 2004). Although we do not know which specific receptors are used to discriminate sex in these experiments, it has been shown that sensory neurons of the VNO have a range of selectivities, with some having as much as 100- or 1000-fold preferences for urinary cues of one sex (Holy et al. 2000). Unless recognition must be achieved over a range of concentrations larger than 100- or 1000-fold, it seems plausible that simple thresholding of female-selective sensory neurons might suffice to recognize sex from urinary cues. Such a model would predict the absence of opponency or any other significant interaction between male and female cues in olfactory sex recognition.

From this perspective, it is therefore remarkable that male cues are synergistic with female cues for triggering vocalizations in naive animals. Although some caution is warranted from the modest number of trials with naive mice, the scale and consistency of the phenomenon is noteworthy. One interpretation of this result is that the cues used by naive animals are indeed present in the urine of both sexes, albeit at somewhat higher concentration in female mouse urine; the addition of male mouse urine to the mixture helps increase its concentration above behavioral threshold. Alternatively, recognition might depend upon the combined detection of multiple cues, at least one which signals sex (and is present largely or exclusively in female mouse urine) and another which signals species information (and is present in urine from both sexes). Such mechanisms may underlie sex recognition in elephants, where the female preovulatory pheromone, Z-7-dodecenyl acetate, requires the presence of other (unknown) sex-nonspecific urinary components for its behavioral effect (Rasmussen et al. 1997). Integration

of cues in olfactory recognition is also required in certain moths, where 2 different female-specific compounds are required to elicit the male pursuit response (Tumlinson et al. 1989); these 2 compounds are detected by different receptor types (Christensen et al. 1995).

In insects, pheromones can trigger courtship behaviors over a wide range of concentrations (Wyatt 2003). This contrasts with the rather restricted range of concentrations that effectively triggered singing in mice (Figure 5). Such a result is not consistent with a scenario in which the concentration of the relevant ligands is in significant excess with respect to their binding to receptors; in such a scenario, the receptors would be fully saturated over a range of stimulus dilutions, and thus, the behavioral response would be insensitive to such dilutions. If the receptors relevant to this task are sensitive to low concentrations of their ligands, as has been reported in some studies of vomeronasal receptors (Leinders-Zufall et al. 2000, 2004; Kimoto et al. 2005), then it suggests that the relevant ligands must be at low concentrations (perhaps nanomolar or below) in urine. However, it also is possible that this task involves receptors that are less sensitive, correspondingly requiring more abundant ligands. In either event, it seems clear that olfactory detection of cues leading to singing is fundamentally different from the ''pursuit'' behaviors used by insects to find mates, in that it depends on female cues at near-full concentrations. This view is also consistent with the mode of odor sampling in the accessory olfactory system, most often thought to require direct physical contact (Wysocki et al. 1982; Luo et al. 2003), for which the ability to perceive identity over orders of magnitude of concentration might be of limited importance.

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