**Application number: XXX**

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**Olfactory modulation of acoustic communication**

**Scientific abstract**

Social communication is highly dynamic, and is modulated by both environmental properties and the needs of the interacting individuals. Therefore, revealing how social and non-social environments affect animal communication is a key question in neuroscience. Tackling this problem is complex brains has been challenging as it often involves the integration of multisensory cues over distributed brain areas. Here we propose to tackle this question by investigating the modulation of acoustic communication in adult *Drosophila* by ethologically relevant, social and non-social cues. Acoustic communication is essential for fly reproductive behavior, which typically occurs in fly-dense environment and over food patches. Therefore, part of the environmental, non-social cues is defined by the landscape of odorants released by food, whereas the social environment is partly defined by co-specific pheromones (social cues). We will leverage these distinct chemosensory signals to dissect the mechanisms by which social and non-social environmental cues modulate acoustic communication (Fig. 1). Acoustic communication in flies includes courtship song production (males) and perception (in females and males), and has fast and slowly varying components. To dissect the mechanisms underlying the modulation of acoustic communication at multiple timescales, we will combine high-resolution behavioral analysis, synaptic-level neural tracing, tools for manipulating and monitoring defined neuronal populations, and computational modeling. This integrative approach, only feasible in a numerical simple nervous system that is highly amenable to genetic manipulations, will lead to the discovery of circuits and neural activity contributing to the modulation of song production and perception. Based on our current knowledge of the mating circuitry, we postulate that chemosesnory modulation of acoustic communication is mediated, at least in part, by sexually dimorphic circuits.

We will first characterize how olfactory cues modulate song production and perception behaviorally. Then, we will measure the neural modulations using a two-photon microscope. Last, we will dissect the underlying circuit by measuring structural and functional connectivity, and will propose a generative model to inform future experiments.

**Detailed description of the research program**

**Scientific background**

Animals live and interact in complex environments. Understanding the neural mechanisms that allow social communication to adapt to changing social and non-social environments is a key challenge in neurobiology. Here we propose to tackle this question using *Drosophila melanogaster* as a model system, taking advantage of the relative simplicity of this system, the available genetic tools, and the current understanding of the circuit underlying social communication in flies. Owing to the fact that acoustic communication is essential for reproduction in *Drosophila* [13,14](https://paperpile.com/c/Q5E40E/yu4g%2Bd59g), and that the fly olfactory system is processing ethologically relevant social and non-social cues (though pheromones [15](https://paperpile.com/c/Q5E40E/sUH8) and food odorants [11,12](https://paperpile.com/c/Q5E40E/BB9q%2BfjfE)), this proposal is focused on olfactory modulation of acoustic communication.

Lab and field studies in multiple model organisms demonstrate that social communication is modulated by social and non-social environmental factors. Examples for social factors include the exposure to rivals [16](https://paperpile.com/c/Q5E40E/esd2), the heterogeneity of the social environment [17](https://paperpile.com/c/Q5E40E/05kL) and the presence of predators [18](https://paperpile.com/c/Q5E40E/tRYR). Examples for non-social parameters include light conditions [19](https://paperpile.com/c/Q5E40E/0eai), temperature [20](https://paperpile.com/c/Q5E40E/Nv2x), and food availability [11,12,21–24](https://paperpile.com/c/Q5E40E/B83e%2BBB9q%2BD1Ng%2B72Xd%2BfjfE%2BqFKi).

While much of the circuit for processing social cues was dissected in model organisms such as mice [] and flies [25,26](https://paperpile.com/c/Q5E40E/sTtIR%2BgldlK), understanding where and how multisensory social and non-social cues are integrated is challenging in complex systems such as primates and rodents. This is since the sensory information is distributed over multiple brain areas whose connectivity is not fully mapped, and since manipulating or measuring brain activity from deep, intact brain tissues is not always possible in large animals. We propose to use *Drosophila* as a model system, leveraging our knowledge of the mating circuitry [25](https://paperpile.com/c/Q5E40E/sTtIR), the available genetic tools [27,28](https://paperpile.com/c/Q5E40E/oqEx%2Bizml), the availability of synaptic-level tracing of the adult brain [29,30](https://paperpile.com/c/Q5E40E/l2nZ%2B213q), and recent tools for fine behavioral quantification of social behaviors [31,32](https://paperpile.com/c/Q5E40E/f1qR%2BL4Rx). During courtship in *Drosophila melanogaster*, male flies undergo a dynamic multimodal courtship display [26](https://paperpile.com/c/Q5E40E/gldlK), while the females respond to courtship song, integrated over multiple timescales [], and make the ultimate decision, to mate or not to mate [33,34](https://paperpile.com/c/Q5E40E/BaUu%2BFBXUL).

Here we choose to focus on olfactory modulation of acoustic communication, based on the following observations: (1) courtship song is important for fly mating (muting the male or deafening the female significantly reduces mating success []), (2) we can quantify male song and the responses of males and females to courtship song over multiple timescales, from tens of milliseconds to many seconds [] (see Fig. xx), (3) the olfactory system carries both social and non social information, through pheromones and other odorants []. Particularly, as flies aggregate on food patches, where they feed, fight and mate [35,36](https://paperpile.com/c/Q5E40E/kaCi%2B6iGI), we will focus on food odorants []. The role of pheromones and food odorants on mating behaviors in male and female flies was demonstrated in a number of studies []. For example the mating frequency in female flies is modulated by the composition of male strains in their surroundings, likely through olfaction [17,37](https://paperpile.com/c/Q5E40E/05kL%2BGqW7), and food odorant were shown to modulate both female receptivity [] and male courtship intensity [] in *Drosophila melanogaster*. We will focus on pheromones [7,9,12,15](https://paperpile.com/c/Q5E40E/sUH8%2BTTnC%2BKB18%2BBB9q) and food odorants who were previously associated with altered mating behaviors, through olfaction (see Table 1). Interestingly, the effect of food odorants on mating behaviors was found to depend on ionotropic glutamate receptors [11,12](https://paperpile.com/c/Q5E40E/BB9q%2BfjfE).

Male courtship song is composed of two major modes, Pulse and Sine []. Song bouts are highly variable [38,39](https://paperpile.com/c/Q5E40E/scKKr%2BNXvD), in part owing to the fact that male song is modulated by dynamic sensory cues from the female [13](https://paperpile.com/c/Q5E40E/yu4g), and by changing internal states [40](https://paperpile.com/c/Q5E40E/zOgH). Recent work suggests that social context modulates male song complexity, though the underlying mechanisms for this modulation are unknown [39](https://paperpile.com/c/Q5E40E/NXvD).

Females respond to courtship song in multiple ways, including ovipositior extrusion [], turning, and slowing [] (see Fig. xx). By recording and parsing the male song [32,41](https://paperpile.com/c/Q5E40E/L4Rx%2B6MaH), and by tracking and quantifying the responses of males and females to courtship song [] (Fig. xx), we are able to quantify how acoustic communication is modulated by external factors over multiple timescales []. Importantly, the modulation of acoustic communication by olfactory cues may be through changes in the persistent internal state of males and females [], for example by changing the level of arousal [] or motivation []. Such changes may modulate the temporal integration of the response to the acoustic signal [] and the persistence of male song [].

Many of the cells involved in the control of mating were shown to express the sex determination factors *doublesex* and *fruitless* (‘*dsx*+’ and ‘*fru*+’ cells; [25,42](https://paperpile.com/c/Q5E40E/sTtIR%2B7gC2K)). This includes neurons who respond to auditory [33,43,44](https://paperpile.com/c/Q5E40E/BaUu%2Bgrtl%2BOM81), olfactory [7,12,33](https://paperpile.com/c/Q5E40E/BB9q%2BBaUu%2BTTnC), visual [45,46](https://paperpile.com/c/Q5E40E/s2vp%2BHpnc) and gustatory [4,47,48](https://paperpile.com/c/Q5E40E/OwsQ%2B3ZJa%2BEXBR) cues, and neurons who are involved in controlling male singing [39,44,49–51](https://paperpile.com/c/Q5E40E/O6LY%2BSC6A%2Bm8KD%2BOM81%2BNXvD). This is critical, as it allows us to focus our search for the underlying on a relatively small and well defined group of cells. We hypothesize the modulation of acoustic communication by social and non-social cues in *Drosophila* is mediated, at least in part, through *dsx*+/*fru*+ cells.

**Research Objectives**

This research proposal focuses on determining how social and non-social olfactory cues - pheromones and food odorants - modulate acoustic communication in *Drosophila*. We will focus on olfactory stimuli for which (1) an effect of mating behavior was already reported, and (2) the reported effect was at least in part through olfaction (3) Olfactory receptors neurons are identified (see Table 1). We will study the olfactory modulation of song production and processing side by side, focusing on *doublesex* and *fruitless* expressing cells (*dsx+*, *fru+*) in the male and female brains.

**Aim 1: Determine how olfaction modulates the response of males and females to courtship song**

Testable hypotheses:

1. Pheromones and food odorants modulate male and female response to courtship song.
2. The effect of pheromones on the response to courtship song is sexually dimorphic.

To test these hypothese we will:

1. Characterize the behavioral response of solitary males and females and of male-female couples to courtship song in the presence and absence of artificially applied stimuli, and in response to activating/ inactivating the corresponding sensory neurons (Table 1).
2. Use a Two-photon microscope to monitor the responses of *dsx+* and *fru+* cells to courtship songs in the presence and absence of artificially applied stimuli.

A successful outcome of this aim will be finding pheromones and food-odorants whose presence modulates both behavioral and neural response to courtship song.

**Aim 2: Reveal how olfaction modulates song production in male flies**

Testable hypotheses:

1. Pheromones and food odorants modulate the structure of male courtship song, specifically - its complexity and persistence.
2. The modulation is both direct (by modulating song-control circuits) and indirect (by modulating male-female interaction, e.g., distance and speeds).

To test these hypothese we will:

1. Characterize the structure of courtship song in solitary and courting males under the presence/absence of pheromones and food odorants. By looking at the two conditions we will be able to differentiate between the direct and indirect modulations.
2. Characterize neural dynamics of *dsx+* neurons triggered by activation of song command neurons in the presence/absence of olfactory stimuli.

Record how olfaction modulates neural dynamics in song-control circuits during and following the activation of song command neurons. We will monitor brain activity in a fixed behaving fly, using a two-photon microscope, while recording fly song.

A successful outcome of this aim will be finding pheromones and food-odorants whose presence modulates male singing, and finding dsx+/fru+ cells whose activity is correlated with the modulation of song by olfactory cues.

**Aim 3: Reveal the circuit and mechanism of olfactory modulated acoustic communication**

Testable hypotheses:

1. Olfactory signals modulate the temporal integration of courtship song processing. Some pheromone induced modulations are sexually dimorphic.
2. Olfactory signals modulated the temporal structure of song bouts and the persistence of male song through sexually dimorphic cells.

To test these hypotheses we will:

1. Derive computational models for olfactory processing of song perception and production, and test/tune the models by testing a wide range of auditory stimuli or activation patterns.
2. Use a combination of neural tracing (using flyWire) and functional imaging to reveal how the olfactory signal is relied to central sexually dimorphic *dsx+* and *fru+* cells.

A successful outcome of this aim will be finding 1-2 odorants whose presence modulates both singing and neural activity in song-controlling cells, in a way that correlates with the singing of the imaged male.

**Expected Significance**

While we know from field work, from our daily experience and from clinical work that social communication relies heavily on environmental context, most lab studies of social communication in the field of neurobiology use isolated pairs. Here we leverage the advantages of *Drosophila* as a model system, as well as recent tools for neural tracing and behavioral quantification, to address a fundamental question in biology: how social and non-social cues from the environment modulate social communication.

In disorders characterized by rigid and/or repetitive behaviors, such as obsessive-compulsive disorder or autistic spectrum disorders, social challenges are common and often hamper sexual relationships[52,53](https://paperpile.com/c/Q5E40E/o995d%2BbGknB). In these disorders, inflexible social behaviors may expose affected individuals to dysfunctional interpersonal contexts. Even subtle limitations in the capability to integrate contextual environmental factors together with innate factors in the context of social and sexual behavior may result in significant dysfunction of marital and social relationships. Furthermore, overly flexible and unstable sexual behavior, as frequently observed in borderline personality disorder[54](https://paperpile.com/c/Q5E40E/V7lz4), also plays a pivotal role in this type of psychopathology.

If successful, this proposal will add a major contribution in closing the gap in our understanding of the underlying mechanisms for context-dependent social communication.

**Experimental design and methods**

**Aim 1: Determine how olfaction modulates the response of males and females to courtship song**

**1.1 Measure how olfaction modulates auditory behavioral response**

We will conduct the behavioral experiments in three settings: (1) solitary males or female, measuring change-in-speed following sound playback [] (2) groups of males, measuring chasing behavior in response to sound playback, (3) a male-female courtship assay, measuring the responses of females to male singing. All setups are high-throughput: we will run 24 flies in parallel in the playback assays (2 assays with 12 flies in each one) , as we previously did ([]), and 6 pairs is 6 courtship assays, similar to ones I previously used [].

In all three setups we will compare the behavioral responses (see Figure xx) when applying an odorant (by coating the chamber ceiling, to minimize contact with the fly legs; Table 1) or not applying any odorant (control). **Playback with solitary flies -** In FLyTRAP [], we will measure the change in speed of solitary males and females in response to auditory stimuli in the presence and absence of odorants (Table 1). In each experiment, we will play Sine (100,150,300 Hz) and Pulse song (IPI = 16, 36, 56ms) using a speaker (presudorandomizing the stimulus order) (Fig xx). We will use a stimulus duration of 4 seconds, and an inter stimulus interval of 1 minute as previously done []. We will compare the auditory response in wild type CantonS males and females, in the presence and absence of physically applied chemical compounds (at the plastic ceiling of the chamber, applied at the beginning of each session). **Playback with Groups of flies -** using the same setup and auditory and olfactory stimuli, we will quantify song-induced male-chaining behavior as previously done [43](https://paperpile.com/c/Q5E40E/grtl). **Courtship assay -** It is possible that some auditory responses depend on other sensory cues or on social context, and we will therefore not be able to reveal them by measuring the response in playback assays. We will therefore also measure the response of females to male song in courtship assay. We will pair a single female with a single males for 15 minutes, measuring the female responses to male song with and without applied odorants (applied on the ceiling before each session). The courtship arena has an array of 9 microphones and a top camera (recording at 150fps), similar to the one we previously used []. Male song will be parsed and the pose of males and females will be estimated using deep-network based tools [31,32](https://paperpile.com/c/Q5E40E/L4Rx%2Bf1qR). We will measure the female response to male song (as well as male singing, see Aim 2) over multiple timescales, from sub-seconds (ovipositor extrusion, turning []; Figure xx) to 10s of seconds (female slowing [], Figure xx).

Preliminary data indicates that the presence of cVA decreases the female response to male song (Fig xx), and that the presence of food enhances copulation probability in CantonS flies (Fig xx).

**1.2 Reveal the role of specific receptors in olfactory modulation of acoustic response**

Once we identify specific pheromones or food odorants that have significant effect on acoustic response in males or females, we will test the role of the corresponding olfactory receptor neurons (Table 1), using known genetic drivers (see Table 1). We will perform both activation and inactivation experiments, using optogenetic manipulations. The choice for using optogenetic inactivation comes from our previous observation that responses in the playback assay are sensitive to the genetic background []. In flies, ATR (all-trans-retinal) is a food supplement that is necessary for the activation of channelrhodopsins in Drosophila, and therefore comparing flies with supplemented ATR in their food (ATR+) versus flies with no ATR supplement (ATR-) is a common practice in Drosophila, both for activation [] and inactivation [] experiments. We will turn the light on (green for inactivation using gtacr1 [] and red for activation using csChrimson []) 8 seconds before the onset of the (4 second) auditory stimuli, and off 8 seconds after the end on the stimuli (so in 1 minute, the light is on for 20 seconds and off for 40 seconds, times 120 trials).

Based on the number of conditions and setups (we have multiple incubators, allowing experiments to run at Zeitgeber 0-3 throughout the working hours), we estimate the data collection phase to last 4 months, including data analysis of the results in aim 1.1, that are necessary for conducting the experiments described in aim 1.2. Based on these results, we will focus on specific stimuli in the imaging experiments (aim 1.3 below).

**1.3 Characterizing olfactory modulation of auditory response in sexually dimorphic cells**
We will test the hypothesis that olfactory modulation of song response occurs in *dsx+*/*fru+* cells by comparing Calcium response on *dsx+*/*fru+* cells to auditory playback under a two-photon microscope in the absence and presence of olfactory stimuli. Based on previous data showing response to courtship song and to cVA in specific *dsx+* populations [33,44,55](https://paperpile.com/c/Q5E40E/BaUu%2BOM81%2BNuUK), we will first use cVA as the olfactory stimulus, and will then expand to *fru+* neurons and to other pheromones and food-odorant based on the behavioral results (Aim 1). Importantly, female *dsx+* neurons in the LPC were shown to response both to courtship song and to cVA [56](https://paperpile.com/c/Q5E40E/WprU), but as multiple dsx+ cells project to the LPC [44](https://paperpile.com/c/Q5E40E/OM81), it is not clear if both signals overlap at the level of individual cells, and if so - which cells.

In each trial we will introduce auditory playback alone, olfactory stimulus alone (presenting the stimuli either mechanically or via air-stream), and a combination of the two. Auditory playback stimuli will include Pulse song (IPI = 16/36/56 ms), Sine song (Frequency = 100/150/300 Hz) and White noise (as in [57](https://paperpile.com/c/Q5E40E/5fIQ)) at different durations, to test the response (tuning, amplitude and temporal integration) of dsx/fru cells to courtship song the presence and absence of different olfactory cues. We will deliver olfactory stimulation in three ways: (1) mechanically, by bringing a soaked paper in close proximity to the fly antena [] using a micromanipulator and a dedicated camera, (2) using air-stream, (3) Using optogenetic activation of specific olfactory receptors of olfactory receptor neurons (Table 1). When using optogenetic activation, we will calibrate the activation protocol based on behavioral results in freely moving flies (Aim 2.1). The choice between delivering through air-stream or mechanically depends on the volatility of each compound. For example, cVA is known to be a ‘sticky’ pheromone, and therefore hard to remove from the delivery system (though cVA was delivered by air in multiple studies, e.g., []). While I had some experience building a system for delivering olfactory stimuli as a post-doc in the Murthy lab, I have limited experience with the design and control of delivering olfactory stimuli. Prof. Moshe Parnas from the Tel Aviv University who has extensive experience in two-photon imaging of olfactory response will help our lab establish the odor-delivery system.

We will conduct further experiments, expanding the set of olfactory stimuli based on our behavioral observations (aims 1.1, 1.2) and to *fru*+ neurons in the LPC and Lateral horn (LH), based on our preliminary data suggesting that there is a broad auditory response in *fru+* cells (Fig xx), including around the LPC and LH, areas who were previously suggested as multisensory hubs [58–60](https://paperpile.com/c/Q5E40E/c8NX%2BYfKH%2BCVzi). The LH is known to process pheromones and food-odors that modulate mating behaviors [12,15](https://paperpile.com/c/Q5E40E/sUH8%2BBB9q). Interestingly, while the *dsx+* auditory cells are all tuned to Pulse song [44](https://paperpile.com/c/Q5E40E/OM81), whole brain activity imaging revealed that auditory responses in the LH are mostly to sine song [57](https://paperpile.com/c/Q5E40E/5fIQ), suggesting a possible olfactory modulation of sine song in LH. Once we identify olfactory modulations of auditory response in LH/LPC *fru*+ cells, we will be able to nail them down to more specific subsets using neural tracing (FlyWire) and existing sparse lines (split-Gal4) for *fru+* cells (as explained in more detail under Aim 3.

**Potential problems and alternative strategies**

It is possible that each pheromone of food odorant alone has weak or no effect, but a combination does. For example, it has been reported that there is a synergetic effect of cVA and vinegar [23](https://paperpile.com/c/Q5E40E/72Xd). In this case we will have to test some mixtures, including the option to run some experiments over food patches with and without added pheromones. It is also possible that the effect of pheromones or food odorants on acoustic communication is solely by gustation (even though, for example, the response of *dsx+*pCd cells to cVA is through olfaction [33](https://paperpile.com/c/Q5E40E/BaUu), and the Table 1 point to olfactory mediated effects only). While this proposal is focused on olfaction, we will consider testing the neural effect mediated by contact as previously done (e.g., [4,48](https://paperpile.com/c/Q5E40E/EXBR%2B3ZJa)). Finally, It is also possible that optogenetic activation will not induce a behavioral effect (on male singing or on song-response), even if the corresponding odor (Table 1) does. In this case, we will not be able to use optogenetics activation for imaging experiments, and will have to introduce the olfactory cue physically (by proximity or using air-stream).

**Aim 2: Reveal how olfaction modulates song production in male flies**

**2.1 Determine how olfaction modulates singing in isolated males.**

We will induce male singing by optogenetically activating the *dsx+* cells P1a and pC2, which drive persistent singing in solitary, freely moving males [44,51](https://paperpile.com/c/Q5E40E/m8KD%2BOM81). We will measure singing with and without the presence of pheromonal or food-derived odorants (using a circular chamber, tiled by 9 pressure microphones as in [31](https://paperpile.com/c/Q5E40E/f1qR)). Song will be parsed (to fPulse, sPulse and Sine) [32](https://paperpile.com/c/Q5E40E/L4Rx) and measured both during the ‘activation period’ (light on) and the persistent period (after stimulus ‘offset’) [61](https://paperpile.com/c/Q5E40E/Dan5) using various activation windows [39](https://paperpile.com/c/Q5E40E/NXvD). We will measure the effect of olfactory cues (see Table 1) on song structure and intensity (Fig xx), to test the hypothesis that song persistence and complexity are modulated by olfactory cues [39](https://paperpile.com/c/Q5E40E/NXvD). As in aim 1.1, we will apply the chemical by coating the chamber ceiling before each experiment. In each experiment we will vary the duration of the optogenetic activation (the ON period) across trials [39](https://paperpile.com/c/Q5E40E/NXvD).

**2.2 Determine how olfaction modulates male singing during courtship**

Using the same dataset collected in Aim 1.2 (male-female pairs), we will measure how pheromones and food-derived odors modulate male song. Following the observation that visual cues modulate song dynamics [13](https://paperpile.com/c/Q5E40E/yu4g) in a state dependent manner [40](https://paperpile.com/c/Q5E40E/zOgH) and that the probability for complex song bouts depends on social context [39,40](https://paperpile.com/c/Q5E40E/zOgH%2BNXvD), we will measure how olfactory cues modulate visually guided song transitions (using GLM filters as done before [13](https://paperpile.com/c/Q5E40E/yu4g)) and song complexity [39](https://paperpile.com/c/Q5E40E/NXvD). Once we identify which olfactory compounds modulate male singing, we will conduct activation and inactivation experiments, as in Aim 1.2, to determine the role of specific olfactory receptor neurons.

**2.3 Characterizing how olfaction modulates singing in a fixed-walking male**

Based on the behavioral findings (aims 2.1, 2.2), and as an intermediate step before the imaging experiment (aim 2.4), here we will characterize olfactory modulation of song in fixed-males, glued to a dissecting chamber, and walking on an air supported ball (Fig xx). We will record male singing using two pressure microphones, positioned in proximity to the fly wings as we already tested (Fig xx). We will use walking flies (and track fly walking using <https://github.com/murthylab/fly-vr> ) as walking state was shown to be correlated with wide-brain dynamics in flies [62](https://paperpile.com/c/Q5E40E/Xgin), and is particularly linked to male singing [13](https://paperpile.com/c/Q5E40E/yu4g). Olfactory stimuli will be presented as described in Aim 1.3.

**2.4 Revealing the neural dynamics underlying olfactory modulation of male singing**

Here we aim to reveal the role of pheromones and food odorants in modulating singing in males. Motivated by recent findings, we will first focus on the pC1 and pCd populations. By activating P1a in a walking fly under a two-photon microscope we will drive persistent male singing [] and persistent activity of *dsx*+ cells including pC1 and pCd [39,55](https://paperpile.com/c/Q5E40E/NXvD%2BNuUK).
Activation of P1a cells (a pC1 subset) drives a persistent behavioral state though a set of recurrently connected cells, that include the cVA responding pCd cells [55](https://paperpile.com/c/Q5E40E/NuUK). pCd cells were found to be necessary for, but not sufficient to trigger, physiologic persistence [55](https://paperpile.com/c/Q5E40E/NuUK). This suggests that cVA activation plays a role in allowing persistent male singing. By activating P1a cells, recording Calcium activity simultaneously in *dsx+* cells and recording male song (using a pressure microphone near each wind, see preliminary data in figure xx) we will test if cVA activation enhances persistent male singing that is locked to persistent activity in the *dsx+* pC1, pCd and pC2 cells. As pC1, pC2 and pCd cells are all doublesex expressing, it is possible to image from these three populations in one fly. With our Resonance scanner, imaging 15 planes to cover pC1, pC2 and pCd cell bodies at a 1um distance between planes is possible at 3Hz (see previous examples for volumetric imaging of *dsx+* cells in my previous works [44](https://paperpile.com/c/Q5E40E/OM81) and [61](https://paperpile.com/c/Q5E40E/Dan5)). Critically, as we are record fly song and neural activity simultaneously (Fig xx), we can correlate the two, finding if specific odorants modulate both the male singing behavior and neural activity in a correlated manner.

**Potential problems and alternative strategies**

It is possible that olfactory modulation is upstream of the cells we use for activation (P1a, pC2), and therefore optogenetic activation may override the effect. Using low activation levels may solve this issue (as the net activity of P1a and pC2 will be due to the upstream modulation and the artificial activation). In this case we will have to use two binary systems (LexA for activation), preferably using non-overlapping integration sites [63](https://paperpile.com/c/Q5E40E/zDO2). It is also possible that the modulation is all downstream the *dsx*+ or pIP10 cells, in which case we will find behavioral but no neuronal correlate for the olfactory modulation of song. In this case we will have to image downstream targets such as pIP10 cells [56](https://paperpile.com/c/Q5E40E/WprU) and P2b [64](https://paperpile.com/c/Q5E40E/RNvW).

**Aim 3: Reveal the circuit and mechanism of olfactory modulated acoustic communication**

We aim to complete Aims 1,2 in two years. Aim 3 is dedicated for revealing the circuit and dynamics, and for deriving theoretical models for olfactory modulation of song production and perception.

Aim 3.1 and 3.2 focus on the processing and production of courtship-song, respectively.

The exact choice of the experiments we will conduct in this aim depend on previous results from aims 1,2, and are therefore described as such.

**3.1 Finding mechanisms, and deriving a model, for olfactory modulation of song processing**

Here we will focus on a small subset of olfactory compounds out of the ones we tested, based on our findings in aim 1. Priority will be given to a single pheromone of food odorant whose effect on auditory processing is sexually dimorphic.

3.1.1 In order to inform a computational model, we will characterize the behavioral and neuronal effects on olfactory cues on song processing using a wider set of song parameters, varying parameters over multiple timescales as previously done (as in [39](https://paperpile.com/c/Q5E40E/NXvD)) with and without the olfactory stimuli. Based on our previous results (from aim 1), we will decide how to deliver the olfactory stimuli.

If we are able to control the temporal pattern of the olfactory stimulation (by using optogenetic stimulation or, in the case of the imaging experiments, also air stream), we will also test how varying the temporal sequence (between olfactory and auditory stimuli) contribute to the olfactory modulation of the auditory responses. This is in order to inform a model for olfactory modulation of auditory responses.

3.1.2 We will derive a computational model for olfactory modulation of acoustic communication (Fig xx). Aims 3.1.1 and 3.1.2 will be conducted side by side - the model will be informed by the experimental results and the choice of the experimental parameters will be in a way that informs the model.

3.1.3 We will use FlyWire to look at the connectivity between the olfactory pathway and the *dsx+*/*fru+* cells whose auditory response is modulated by olfaction. We will find candidate intermediate cells. However, as the fly brain is highly interconnected, indirect paths may include many candidates []. In the case that there are strong candidates, we will build sparse lines and test functional connectivity. Here we will take a similar approach to one we took in a recent work (a student I mentored at the Murthy lab; manuscript in preparation), where we identified visual inputs to an ovipositor-extrusion command neuron [34](https://paperpile.com/c/Q5E40E/FBXUL) in FlyWire, built a split line (using NeuroBridge []) and found functional connectivity by combining optogenetic activation and two-photon Calcium imaging. For testing functional upstream dsx+/fru+, we will cross the following lines I already created and tested [61](https://paperpile.com/c/Q5E40E/Dan5), crossed to the relevant split-gal4 lines:
1. *10xUAS-Chrimson.tdTomato,13LexAop2-GCaMP6s/+;Sp/CyO;dsx-LexA/TM6B,tb*

2. *10xUAS-Chrimson.tdTomato,13LexAop2-GCaMP6s/+;Sp/CyO;fru-LexA/TM6B,tb*

3.1.4 We will conduct activation and inactivation experiments to find if specific groups (of *dsx+*/*fru+* cells or intermediate cells that we found in aim 3.1.3) are necessary and sufficient for olfactory modulation of behavioral/neural song response.

**3.2 Finding mechanisms, and deriving a model, for olfactory modulation of song production**

Here we will focus on a small subset of olfactory compounds out of the ones we tested, whose presence modulates singing and neural activity which is correlated with singing.

Aims 3.2.1-3.2.4 are parallel to aims 3.1.1-3.1.4, except that the latter are focused on song production.

3.2.1 In order to inform a computational model for olfactory modulation of song production, we will record song in males using a larger set of stimuli, optogenetically activating P1a or pIP10 to drive singing [39](https://paperpile.com/c/Q5E40E/NXvD), in the presence and absence of olfactory cues in freely moving solitary males.

3.2.2 We will use the framework recently developed by Roenschied et al. [39](https://paperpile.com/c/Q5E40E/NXvD) to model the effect of olfactory stimulation on song production. In the model suggested by Roemschied et al., the transition from ‘simple bouts’ to ‘complex bouts’ is driven by social context, possibly in part based on gustatory and visual cues. Here we will measure the effect of olfaction on song patterning over a range of stimuli (3.1.1) as in Roemschied et al., 2021, testing the hypothesis that olfactory cues contribute to song patterning, either by signaling the presence of other flies (by pheromones), or the presence of food (by food odorants). Based on mour model predictions, we will choose a small set of stimuli, for which we will simultaneously record fly singing and Calcium response in *dsx+* cells as explained in aim 2.4.

3.2.3 Here we aim to find connectivity from specific odorant receptors to song command cells whose activity is modulated by olfaction in a way that is correlated with the effect on song (see aim 2.4). The connectome of the male brain is expected to be released during 2023, while Aim 3 of this proposal is planned only for the third year of the project, therefore starting October 2025. We will use the male-brain connectome to look at the connectivity between the relevant olfactory neurons and the song-controlling cells whose activity was modulated by a specific odorant.

3.2.4 Depending on the number of candidate cells in the pathway, we will consider building specific split-Gal4 lines, and test functional connectivity with P1a and pC2, as well as how optogenetically activating the intermediate cells impact the olfactory song modulation. This will allow us to tell if a given cell is (1) functionally upstream P1a and/or pC2, (2) is necessary for olfactory song modulation.

The expected outcome of Aim 3 is to have circuit diagrams and models for olfactory modulation of song perception and production.

**Potential problems and alternative strategies**

The modulation of song production by olfactory signals may be happening in the ventral nerve cord (VNC), in which case we will have to choose between imaging in the VNC, or only having a behavioral description. While two-photon imaging in the VNC is feasible [65](https://paperpile.com/c/Q5E40E/21BQ), the author of this proposal has no previous experience in imaging from the VNC. In this case my lab will contact Prof. Pavan Ramdya (EPFL) to ask for technical guidance. Both PIS know each other and corresponded in the past.

**Closing remarks and outlook**

[still missing]

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