**Application number: XXX**

**PI: David Deutsch**

**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 in complex brains, however, has been challenging as it often involves the integration of multisensory cues over distributed brain areas. Here we propose to address 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 environments and over food patches. Environmental non-social cues are thus largely 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 components that vary rapidly and slowly. 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 simple nervous system that is highly amenable to genetic manipulation, will lead to the discovery of circuits and neural activity that regulate song production and perception. Based on our current knowledge of the mating circuitry, we postulate that the chemosensory 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. Lastly, 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 settings is a key challenge in neurobiology. Here we propose to tackle this question in *Drosophila melanogaster*, taking advantage of the relative simplicity of this model system, available genetic tools, and the current understanding of the neural circuitry underlying social communication in flies. As acoustic communication is essential for reproduction in *Drosophila* [13,14](https://paperpile.com/c/Q5E40E/yu4g%2Bd59g), and the fly olfactory system processes 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 the olfactory modulation of acoustic communication.

Lab and field studies in multiple model organisms demonstrate that social communication is influenced by social and non-social environmental factors. Relevant social factors include 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), while non-social factors 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 neural circuitry involved in the processing of social cues has been 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 because this sensory information is distributed over multiple brain areas whose connectivity is not fully mapped, and manipulating or measuring brain activity from deep, intact brain tissues is not always possible in large animals. Here, propose to use *Drosophila* as a model, leveraging our knowledge of their mating circuitry [25](https://paperpile.com/c/Q5E40E/sTtIR), established 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 recently established tools for the fine behavioral quantification of social behaviors [31,32](https://paperpile.com/c/Q5E40E/f1qR%2BL4Rx). During courtship, male *Drosophila melanogaster* exhibit a dynamic multimodal courtship display [26](https://paperpile.com/c/Q5E40E/gldlK), while the females respond to courtship song, integrated over multiple timescales [], and ultimately decide to mate or not to mate [33,34](https://paperpile.com/c/Q5E40E/BaUu%2BFBXUL).

Here we choose to focus on the 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), and (3) the olfactory system carries both social and non-social information through pheromones and other odorants []. Given that flies aggregate on food patches where they feed, fight, and mate [35,36](https://paperpile.com/c/Q5E40E/kaCi%2B6iGI), we will specifically focus on food odorants []. The role of pheromones and food odorants in the shaping of the mating behaviors of male and female flies has been demonstrated in many studies []. For example, female fly mating frequency 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 odorants have been shown to modulate both *Drosophila melanogaster* female receptivity [] and male courtship intensity []. We will focus on pheromones [7,9,12,15](https://paperpile.com/c/Q5E40E/sUH8%2BTTnC%2BKB18%2BBB9q) and food odorants that have previously been 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 because 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 ovipositor 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 thereto [] (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 have been 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 that 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, as well as neurons 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 circuitry 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 has already been reported, (2) the reported effect was at least in part mediated through olfaction, and (3) olfactory receptor neurons have been 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. To achieve these goals, we propose the following specific aims:

**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 responses to courtship song.
2. The effect of pheromones on the response to courtship song is sexually dimorphic.

To test these hypotheses we will:

1. Characterize the behavioral response of solitary males and females and 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 for this aim will be the identification of pheromones and food odorants, the presence of which modulates both behavioral and neural responses 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 hypotheses 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 direct and indirect modulation.
2. Characterize the neural dynamics of *dsx+* neurons triggered by activation of song command neurons in the presence/absence of olfactory stimuli.
3. 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 for this aim will include the identification of pheromones and food odorants, the presence of which modulates male singing, and the identification *dsx+/fru+* cells whose activity is correlated with the modulation of song by olfactory cues.

**Aim 3: Reveal the circuits and mechanisms underlying 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 regulate 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 the olfactory processing of song perception and production, and test/tune these 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 signals are relayed to central sexually dimorphic *dsx+* and *fru+* cells.

A successful outcome for this aim will be the identification of 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 fieldwork, clinical work, and our daily experience that social communication relies heavily on environmental context, most lab studies of social communication in the field of neurobiology use isolated pairs. Here, we propose to leverage the advantages of *Drosophila* as a model system and associated neural tracing and behavioral quantification tools 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 to the ability 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 is 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, the major contribution of this proposal to the field will be the closure of this gap in our understanding regarding the underlying mechanisms governing 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 responses**

We will conduct the behavioral experiments in three settings: (1) solitary males or females, 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 per assay as in our prior work []), and 6 pairs in 6 courtship assays, similar to our previous approach [].

In all three setups, we will compare 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, 56 ms) 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 in previous studies []. We will compare auditory responses in wild-type Canton-S males and females in the presence and absence of chemical compounds physically applied to the plastic ceiling of the chamber 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 done previously [43](https://paperpile.com/c/Q5E40E/grtl). **Courtship assay -** It is possible that some auditory responses depend on other sensory cues or 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 responses of females to male song in courtship assays. We will pair a single female with a single male 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 contains` an array of 9 microphones and a top camera (recording at 150 fps), 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 tens of seconds (female slowing [], Figure xx).

Preliminary data indicate that the presence of cVA decreases the female response to male song (Fig xx) and that the presence of food enhances copulation probability in Canton-S flies (Fig xx).

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

Once we identify specific pheromones or food odorants that have a significant effect on acoustic responses in males or females, we will test the role of the corresponding olfactory receptor neurons using known genetic drivers (see Table 1). We will perform both activation and inactivation experiments via optogenetic manipulation. The choice of an optogenetic inactivation approach stems 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, and comparing flies fed supplemental ATR (ATR+) to those without ATR supplementation (ATR-) is a common practice in *Drosophila* research 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 of the stimuli such that 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 that the data collection phase will last for 4 months, including data analysis of the results of 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 the olfactory modulation of auditory responses in sexually dimorphic cells**
We will test the hypothesis that olfactory modulation of song response occurs in *dsx+*/*fru+* cells by comparing the Calcium response of these cells to auditory playback under a two-photon microscope in the absence and presence of olfactory stimuli. Based on previous data showing responses to courtship song and 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 odorants based on the behavioral results (Aim 1). Importantly, female *dsx+* neurons in the LPC have been shown to respond both to courtship song and 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 are involved.

In each trial, we will introduce auditory playback alone, olfactory stimulus alone (presenting the stimuli either mechanically or via air stream), or 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 in 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 antennae [] using a micromanipulator and a dedicated camera, (2) via air stream, and (3) through the 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 these stimuli via 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 has been delivered by air in multiple studies, e.g., []). While I have some experience building a system for the delivery of 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 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 in which we expand the set of olfactory stimuli based on our behavioral observations (Aims 1.1, 1.2) and will extend these analyses 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 that 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 the possible olfactory modulation of Sine song in the LH. Once we better clarify the olfactory modulation of auditory responses in LH/LPC *fru*+ cells, we will be able to link them to more specific subsets using neural tracing (FlyWire) and existing sparse lines (split-Gal4) for *fru+* cells (as detailed in Aim 3).

**Potential problems and alternative strategies**

It is possible that each pheromone of food odorant alone will have no effect or only a weak effect, whereas a combination may exhibit a stronger effect. For example, it has been reported that there is a synergetic effect of cVA and vinegar [23](https://paperpile.com/c/Q5E40E/72Xd). If that is the case, we will need 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 mediated 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 Table 1 points only to olfactory-mediated effects). While this proposal is focused on olfaction, we will consider testing the neural effect mediated by contact as done previously (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 song responses, even if the corresponding odor (Table 1) does. In this case, we will not be able to use optogenetic activation for imaging experiments, and will instead have to introduce the olfactory cue physically (by proximity or via 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 test chemicals 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), we will characterize the 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 have already tested (Fig xx). We will use walking flies (and track fly walking using <https://github.com/murthylab/fly-vr> ), as the walking state has been 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 the olfactory modulation of male singing**

Here, we aim to reveal the role of pheromones and food odorants in the modulation of 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 through a set of recurrently connected cells that include the cVA-responding pCd cells [55](https://paperpile.com/c/Q5E40E/NuUK). pCd cells have been 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 and simultaneously measuring Calcium activity in *dsx+* cells and recording male song (using a pressure microphone near each wing, 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 these three populations in one fly. With our Resonance scanner, imaging 15 planes to cover the pC1, pC2, and pCd cell bodies at a 1um distance between planes is possible at 3 Hz (see previous examples for volumetric imaging of *dsx+* cells in our previous work [44](https://paperpile.com/c/Q5E40E/OM81),[61](https://paperpile.com/c/Q5E40E/Dan5)). Critically, as we will record fly song and neural activity simultaneously (Fig xx), we can correlate the two, revealing whether specific odorants modulate both 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 that we will target for activation (P1a, pC2), and that optogenetic activation may thus override the effect. Using low activation levels may solve this issue, as the net activity of P1a and pC2 will be due to upstream modulation and artificial activation. In that 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 of the *dsx*+ or pIP10 cells, in which case we will identify behavioral but not neuronal correlates 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 circuits and mechanisms underlying olfactory-modulated acoustic communication**

We aim to complete Aims 1 & 2 in two years. Aim 3 is dedicated to revealing the circuit and dynamics, and to deriving theoretical models for olfactory modulation of song production and perception. Aims 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 will depend on previous results from Aims 1 & 2, and are therefore described as such.

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

Here we will focus on a small subset of the tested olfactory 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 of olfactory cues on song processing using a wider set of song parameters, varying parameters over multiple timescales (as in [39](https://paperpile.com/c/Q5E40E/NXvD)) with and without the olfactory stimuli. Based on our previous results (Aim 1), we will decide how to most appropriately 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, via air stream), we will also test how varying the temporal sequence between olfactory and auditory stimuli contributes to the olfactory modulation of the auditory responses. This will inform a model of the olfactory modulation of auditory responses.

3.1.2 We will derive a computational model for the 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 inform the model.

3.1.3 We will use FlyWire to examine the connectivity between the olfactory pathway and the *dsx+*/*fru+* cells whose auditory response is modulated by olfaction, enabling the identification of candidate intermediate cells. However, as the fly brain is highly interconnected, indirect paths may include many candidates []. If there are strong candidates, we will build sparse lines and test functional connectivity. Here we will take a similar approach to one that we employed recently (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. To test functional upstream *dsx+/fru+*, we will cross the following lines that we have already created and tested [61](https://paperpile.com/c/Q5E40E/Dan5) with 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 were identified in Aim 3.1.3 are necessary and sufficient for the olfactory modulation of behavioral/neural song responses.

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

Here we will focus on a small subset of the tested olfactory compounds, the presence of which modulates singing and neural activity which is correlated with singing. Aims 3.2.1-3.2.4 will be performed in parallel with Aims 3.1.1-3.1.4, with the latter being focused on song production.

3.2.1 In order to inform a computational model for the 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 that model framework, 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 (Aim 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 (through pheromones) or the presence of food (through food odorants). Based on our model predictions, we will choose a small set of stimuli for which we will simultaneously record fly singing and Calcium responses in *dsx+* cells as detailed in Aim 2.4.

3.2.3 Here we aim to detect 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 in 2023, while Aim 3 of this proposal is planned only for the third year of the project, thus commencing in October 2025. We will therefore use the male-brain connectome to examine 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 will test functional connectivity with P1a and pC2, as well as how optogenetically activating the intermediate cells impacts olfactory song modulation. This will allow us to determine if a given cell is (1) functionally upstream of P1a and/or pC2 and (2) is necessary for olfactory song modulation.

The expected outcome of Aim 3 is the establishment of circuit diagrams and models for the 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 scenario, we will contact Prof. Pavan Ramdya (EPFL) to ask for technical guidance. Both PIs know one another and have corresponded in the past.

**Closing remarks and outlook**

[still missing]

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