# Is *Drosophila* song amplitude structure a communication signal?




Birgit Brüggemeier

Magdalen College

University of Oxford

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# Abstract

**Is Drosophila song amplitude structure a communication signal?**
Birgit Brüggemeier
Magdalen College
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*Drosophila* courtship song has been studied for over 60 years and remains an area of active research today. Several studies have investigated the physiological mechanisms for fly song production, but no unifying account exists. We review fly song production and integrate published data to a mathematical model of courtship song production.

We hypothesize that muscle dynamics underlie fluctuations in the amplitude of courtship song. Our model suggests that these fluctuations can be measured and we introduce the term '*song amplitude structure*' (SAS) for those measurements. We predict that SAS signals muscle power and we validate our prediction in muscle mutants of the *Drosophila myosin light chain* (*Dmlc2*) gene.

We then investigate whether SAS is a communication signal in *Drosophila*. We show that the two species *D. melanogaster* and *D. simulans* differ in their SAS and that both females and males behaviourally discriminate their species SAS from other SAS. This suggests that SAS is a communication signal in *Drosophila*.

Perception of SAS may be affected by noise and we therefore examine the effect of noise on responses to SAS. We find that female auditory responses are not impaired by noise in SAS, however male auditory responses are impaired by noise in SAS. This suggests that males and females may be processing noise in SAS differently. Future work should investigate whether sexually dimorphic auditory neurons respond differently to noise in SAS.

We hope that our work will be helpful for investigating fluctuations in amplitude of fly song. We also wish that researchers can use our work for studying the mechanisms underlying both the production of SAS and the perception of SAS.

To my family.

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# Statement of originality

The material I present in this thesis has—to the best of my knowledge—never been published nor has it been written by another person than me except where I explicitly acknowledge so. In Chapter 7 I reprint some figures from published articles. I have received permission by authors of these articles to do so and I explicitly state in captions when I reprint figures.

# List of Acronyms

The following list contains acronyms we use throughout this thesis. The acronym is in the first column and we write it out in the second column of the list.

| | |
|---|---|
| AP | Action potential |
| ABC-SMC | Approximate bayesian computation using sequential Markov chains |
| AMD | Amplitude modulation with distance |
| AMMC | Antennal mechanosensory and motor center |
| CPG | Central pattern generator |
| CPP | Cycles per pulse |
| Dmlc | *Drosophila* myosin light chain |
| cVA | 11-cis-vaccenyl acetate |
| IFM | Indirect flight muscles |
| IPI | Inter-pulse interval |
| IQR | Inter-quartile-range |
| JO | Johnston organ |
| SAS | Song amplitude structure |
| WR | Two-sided Wilcoxon signed-rank test |

# Work associated with DPhil

**Journal articles associated with this DPhil which have been published:**

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# Chapter 1

# Introduction

## 1.1 Animal communication.

"One cannot not communicate"[1] remarked the communication theorist Paul Watzlawick, thereby stressing the omnipresence of his area of research. Communication entails a sender, a message and a receiver[2] and this characterisation is versatile. The sender can be me, the message can be what I write, and the receiver can be you, the reader. The sender doesn't need to be human: Other animals communicate also. For example dogs wag their tails which can be a signal of joy. Messages can be multisensory and make use of one or multiple senses.[1] Seeing, hearing, smelling, touching, tasting can be means to communicate.[1] Thus a dog may whack it's tail, which is a visual signal, or bark, which is an auditory one. They may smell another dog or lick and taste their owner's face and we may touch them and feel their fur to be soft or rough. Such sensory information can be interpreted as messages on the state of the sender and used to chose appropriate responses. In our thesis we focus on auditory messages, in particular fly song, which we introduce in Section 1.4.

Messages can be voluntary or involuntary.[1] Lets consider an example in which the message is that the room temperature is high. This message can be conveyed voluntarily by saying: "The room temperature is high." or it can be sent involuntary by sweating. Note that the motivation of the message may be the same: a high room temperature. However the messages differ in their degree of volition. While the former is deliberate, the latter may be an uncontrolled bodily reaction to heat.

However a receiver may deduce the same action, which may be to open a window, and to let in fresh air. Our thesis discusses an involuntary communication signal, which we call song amplitude structure (SAS) and introduce in Chapter 2.

Receivers may not parse messages correctly. Indeed misunderstandings and deceptions are common throughout the animal kingdom[3–8] and animals can use deception for their advantage: for example hoverflies deceive predators with their yellow-black apparel that is reminiscent of a stinging wasp. Hoverflies do not sting, but their appearance can discourage animals from eating them, thus increasing their survival chances.[7,8] Accurate interpretation of signals may be advantageous, as being deceived can be detrimental. A bird that mistakes a hoverfly for a wasp may miss out on a meal and it is in the interest of the receiver to avoid this. Signals can be corrupted not only by cheating senders, but also by environmental noise. Noise is differentiated into biotic noise (like vocalisations of animals) and abiotic noise (like wind, running water, and traffic noise) and can corrupt communication.[3–6] We discuss in Chapters 5, and 6 how noise can corrupt SAS.

## 1.2 Communication in courtship.

Animals can communicate their willingness and suitability to mate in courtship. In many species males actively court females, and females make mating decisions based on male displays.[9–11] One species in which females are courted by males are peacocks, and males of this species are famous for their flamboyant feathers, which they use to woo females. Growing these feathers is energy costly and moving with them is difficult and can be dangerous, as when the male peacock is attacked by a predator. Long feathers can impede a swift exit, as they are heavy and bulky. In addition the intense colours of the feathers may attract females, but also predators. Thus the mating display of the peacock's tail has been described as handicap. Overcoming this handicap, is indirectly communicating fitness and surviving despite the weight of the feathers is and indirect sign of strength.[7,12] Females may be interested in males that can handle handicaps, as mating with them may give their offspring an advantage in survival.

Courtship communication is energy costly and can take many forms like handicaps, fighting, dancing and calling.[13] These behaviours can signal advantageous characteristics such as health, agility and strength, which may allow females to make mating decisions. However as mentioned in Section 1.1 animals can deceive one another,

thus corrupting the trustworthiness of a communication system. This can initiate an evolutionary arms race for senders to deceive and for receivers to recognise deception,[8] which is regarded as evolutionary unstable. In contrast honest signalling is considered evolutionary stable. Honest signals give accurate information about the states of animals: For example the peacock tail conveys that it's owner was able to survive despite the handicap.[12] Another example for honest signalling can be mating calls. Thus vocal signals enable toads to estimate proximity, size and fighting ability of conspecifics.[6] Lower frequency calls might allow female toads to choose larger males.[6] Mating calls may convey size and strength in birds also. Female starlings might be able to assess the muscle characteristics of males from their song. Superfast vocal muscles control song production in these birds and muscle activity modulates their song amplitude.[14] Female starlings might process amplitude to make mating decisions. Females from other species including mammals,[9] frogs[6] and crickets[5] prefer males with high amplitude calls. In Chapter 2 we formulate the hypothesis that SAS signals muscle power in fruit flies. Then we test our hypothesis in Chapter 3 and show in Chapter 4 that females make mating decisions based on SAS.

Communication in courtship can be species-specific, that is the mating message is specifically produced for conspecifics, who can decode the message. Messages from other species may not be decoded accurately. Such species-specific communication may be accompanied by specialisation of communication devices of the sender and the receiver of messages. Thus fruit flies have evolved ears that resonate at frequencies produced by the wings of their species.[15] We suggest in Chapter 4 that SAS may be species-specific and discuss in Chapter 7 what experiments could test species-specificity of SAS.

## 1.3 Why the fly?

Fruit flies (*Drosophila*) are ubiquitous around the world[16] and scientists have studied flies for more than hundred years.[17] Today *Drosophila* is one of the most widely used model organisms on the world[17] and many tools exist to dissect mechanisms underlying the complex social behaviour of *Drosophila*.[18–20] For example through the use of genetic tools, it is possible to narrow down physiological mechanisms which are responsible for specific behaviours[21] and an unprecedented genetic toolkit exists for working with fruit flies.

In addition fruit flies are relatively inexpensive to keep, which makes them a cost-efficient alternative to other animal-models, like mice, birds and monkeys.[22] These animal models require prior review and approval by governmental institutions in many countries, which can delay experimentation and requires administrative work. Experimentation with flies is exempt by such governmental regulations, and researchers thus save time.

Moreover flies reproduce rapidly: flies take up to a week to sexually mature and can then mate and lay eggs every four to ten days.[23] Females lay hundreds of eggs throughout their life span, which suggests that offspring is abundant. The reproductive rates of other animal models are lower, which can mean higher costs and longer waiting times for experiments.[22,24]

What is more, *Drosophila* is a diverse genus with about 4000 species[23] distributed throughout the world, which makes fruit flies a rich model organism for evolutionary studies.

## 1.4 Courtship song in fruit flies.

*Drosophila* males court females of their species by exhibiting courtship behaviours that include following the female, licking her genitalia and singing.[11] Fruit fly males sing to females by vibrating one of their wings.[25] The song of *D. melanogaster* is structured and they can produce two types of song: sine song and pulse song.[26] Sine is lower in amplitude and lower in frequency than pulse song[26] (see Figure 1.1a for an illustration of sine and pulse song). Pulse increases female receptivity, and sine also increases female receptivity,[27] however less so than pulse.[28] In this thesis we focus on pulse, similar to other researchers.[29–31] Note, that several *Drosophila* species do not produce sine song and the function of sine song is not well understood.[26] Pulse seems more ethologically relevant than sine in increasing female receptivity.[28] Additionally pulse can be reliably detected in an automated way, whereas sine song is not reliably detected automatically,[32] but see[33] for a critical review of automated analysis of song. We discuss sine in Section 1.5.4 and in Chapter 2.

Fly songs are multivariate and courtship songs differ between fly species, having species-specific features: song frequency, inter-pulse interval (IPI), and cycles per pulse (CPP) differ across *Drosophila* species[15,34] and females are able to process differences in song and mate to song of their own species more often than to songs of

other species,[29,35,36] which suggests courtship song acts as mating signal and influences reproductive choices. Attempts exist to dissect male song production,[30,37,38] which show that sine and pulse in *Melanogaster* are functionally independent structures. In contrast song features like frequency, IPI and CPP can not be fully parsed,[30] which suggests that these features may be functionally dependent.

*Drosophila melanogaster* males control and modulate their song depending on sensory cues from the female,[31,39,40] which suggests flexible control of song. However flies do not learn to sing, but are born with the ability to produce a species-specific courtship song: males that are raised in isolation, never heard song, and thus could not have learned how to sing can produce the song that is characteristic of their species and are successful in mating females,[41] which suggests an innate ability to sing. Although the ability to sing is innate, males can flexibly adjust their singing during courtship through sensorimotor transformations.[31] In fact, Coen and colleagues showed that male flies can adjust the amplitude of their song depending on the distance to a female, singing louder when a female is farther away, suggesting amplitude modulation with distance (AMD), an ability that is also possessed by mammals and birds.[3,42]

Fly males visually assess size, form, position, and movement of a target female and adjust their song according to these cues.[31] These sensory cues are then transformed into motor commands which then activate thoracic muscles that control wing vibration and song amplitude.[31,40] This active sensorimotor control of song amplitude may provide relevant information about the singer, as song amplitude correlates with activation level of muscles and muscle power.[31,43] For a discussion see Chapter 3.

It is not clear whether female flies can parse the information from song amplitude, as the received amplitude is likely to be distorted due to several factors, including the rapid attenuation rate of air particle velocity, the position of the singer, male–male competition and environmental noise sources like wind.[4,44,45] Males compensate for some noise by singing louder when more distant to a female,[31] but how do fly males control song amplitude? Additionally, how does the fly ear process song amplitude? We will review control of song amplitude in Sections 1.5 and 1.6 and briefly discuss auditory processing of song amplitude in *Drosophila* in Section 1.7.

## 1.5 How do male flies modulate song amplitude?

### 1.5.1 How does sensory input shape song amplitude?

Flies use their senses to shape their courtship song.[31,39,40] A male fly starts courting when he touches and tastes a female,[46,47] which suggest that close contact between male and female may trigger singing and perhaps could be a prerequisite for song amplitude control. Once a male sings, vision seems to be the predominant sensory modality involved in the control of song amplitude:[31] males sing louder when a female is farther away and thus males may compensate for the distance to a female by singing loud. Additionally, blind males sing significantly louder than controls with intact vision,[31] suggesting that blindness affects the ability of a male to downregulate the loudness of his singing.

Surprisingly however, motion- and loom-sensitive visual circuits (lamina, medulla, lobula, lobula plate) are not necessary for song amplitude control,[31] which suggests that these visual circuits do not control song amplitude. However silencing these visual circuits renders flies only partially blind, and flies may need to be completely blind to interrupt vision control of song amplitude. However blind males exhibit residual amplitude modulation with distance (AMD) when a female is less than about two fly body lengths away (two fly body lengths are about 5 mm),[31] which suggests that AMD at close distances is controlled by sensory modalities other than vision. However, neither males that are unable to smell nor deaf males sing at intensity levels that are significantly different from controls,[31] suggesting that neither olfaction nor audition disrupts amplitude control. What can explain the residual amplitude control in blind males at close distances? Coen et al.[31] suggested that AMD may rely on as-yet-unidentified visual circuits and streams, although this has not yet been tested.

Song amplitude control is linked to sensory cues from the female, and in addition to male movement,[31,39,40] which suggests that male movement may be involved in the modulation of song amplitude. A potential mechanism that could explain a link between male movement and male song amplitude is common activation of thoracic muscles:[48–50] song amplitude correlates with the activation of the thoracic indirect flight muscles (IFM),[31,51] and locomotion correlates with IFM functionality,[49,50] with flies moving less when their IFM are dysfunctional. Male movement might explain the reduction in song amplitude at close distances to the female that can not be explained by sensory processing:[31] the male slows down and stops when getting closer to the female (own observation), however this has to our knowledge not been quantified. If

slowing down requires reduction of IFM activity, and if the male slows down when close to the female, then male speed might explain the amplitude modulation at close distances that can not be explained by sensory processing.[31]

### 1.5.2 Where in a male brain are visual stimuli integrated?

We show in Figure 1.1b a schematic of neurons controlling courtship song. Note that sensory cues during courtship are integrated in a central brain neurons, known as P1, which are involved in song control.[30] Neuronal projections from P1 overlap with input sides of pIP10 neurons that project to the thorax. In the thorax some circuits may act as central pattern generators (CPG) for courtship song (dPR1, vPR6, vMS11,[30] and TN1A[38]). These CPGs may control different aspects of song.[21,30,38] Visual cues might integrate at the level of thoracic CPGs to control song amplitude,[31] as artificially activating thoracic CPGs renders flies unable to control their amplitude with distance, while when pIP10 and P1 neurons are artificially activated male flies control their amplitude with distance normally. Consequently, Coen and colleagues suggest that visual neurons project to thoracic CPG and artificial activation of thoracic CPGs disrupts integration of visual cues.[31] However artificial activation of thoracic CPGs disrupt song in general:[30] song parameters as inter-pulse interval (IPI), song frequency and cycles per pulse are more disrupted when activating thoracic CPGs than when activating upstream P1 or pIP10.[30] Hence, it may be possible to explain the observation that artificial activation of thoracic CPG disrupts amplitude modulation with distance by the general disruptive effect of artificially activating thoracic CPG in *Drosophila*. It is not currently known where vision neurons connect to the song control circuit and it is necessary to conduct further anatomical, physiological and behavioural studies to determine (1) which vision neurons are involved in song control and (2) where these vision neurons integrate in the song control circuit[1].

### 1.5.3 Which muscles and neurons control song amplitude?

Muscle activity seems to play an important role in controlling song amplitude.[31,43] In Figure 1.1c we show a schematic of thoracic muscles that may be involved in song control. The frequency of neuronal firing on the indirect flight muscles (IFM),

[1]There is unpublished anatomical evidence that primary visual neurons overlap with interneurons that connect to pC1 neurons,[52] which suggests that there may be a connection between primary visual input and the pC1 integration center.

which consist of the dorsal longitudinal muscle fibers (dlm) and the dorso-ventral muscle fibers (dvm), correlates positively with song amplitude:[31] the more the IFM motorunits fire, the louder the fly sings, which suggests a positive relationship between song amplitude and neuronal activity of motorneurons. However it has not been tested whether this positive relationship between neural firing rate and song amplitude occurs in neurons upstream of IFM motorneurons. Neurons that may control song amplitude are the thoracic dPR1, vPR6, vMS11 and TN1A or the projection neurons pIP10, or the central brain neurons P1. Artificial activation of these neurons at different intensities affects song features as song frequency, inter-pulse interval (IPI) and cycles per pulse (CPP),[30] but the effect of stimulation intensity on song amplitude has not been tested.

Song amplitude correlates with neural activation in IFM motorneurons, which suggests that song amplitude signals activation of motorneurons. If song amplitude signalled also activation of the song control circuit,[30] it would suggest that song amplitude could convey information about the excitation status of males when singing, which would be relevant information for both female flies and male competitors. To test whether song amplitude correlates with activation of the song control circuit one could record activity in candidate neurons in males[30] while at the same time recording courtship song of these males.[18,40]

### 1.5.4 Is sine song amplitude modulated?

Amplitude modulations have been reported and studied in pulse song,[31,51] but do amplitude modulations exist in the humming element of fly courtship song, in sine song? Sine song amplitude modulations have been reported[51] and muscle activity has been suggested as origin for sine song amplitude modulations,[51] which is in agreement to the role of muscle activity in pulse song amplitude modulation.[31] However sine song is quieter than pulse song[26] and at the time sine song modulations were reported (1977) sine song was hardly picked up by microphones,[51] which puts into question whether the reported modulations in sine amplitude have a biological origin, or rather reflect noisy recordings. However our own observations of recordings made with modern microphones make me believe that sine song modulations exist.

The hg1 muscle is necessary for sine song production[37] (see Figure1.1c), and thus is an interesting target to investigate potential sine song amplitude modulations. To test whether hg1 modulates sine song amplitude, the hg1 motor neuron can be artificially activated to drive activation of the hg1 muscle,[37] which may control song

amplitude of sine song. Note, however, that other muscles (baslar muscles and large indirect flight muscles) are active when male flies sing sine song,[48] which suggests that muscles other than hg1 may be involved in sine song amplitude control. The study of sine song is difficult because of the low mean intensity of sine song[26,31,51] and the lack of reliable automated methods to detect sine in song recordings.[32] Thus a comprehensive analysis of sine song amplitude modulation may require improvements in recording techniques and song analysis software.

## 1.6 What else influences song amplitude?

While vision and muscle power play significant roles in song amplitude generation,[31,43] other factors also influence song amplitude; these include wing size[31,54] and certain mutations.[55–57] Males with large wings sing louder than males with short wings[31,54] and are more successful in mating with females than males with short wings.[54] Note that the larger a male, the larger his wing[54] and the more successful he is at mating with females.[58] The mating success of large males may be due to their singing louder than small males,[54] to their being stronger than smaller males, or both.

Song amplitude is affected by several mutations (*cacaphony* (*cac*),[55–57,59] *dissonance* (*diss*),[55,60] and auditory mutations *atonal* (*ato*), *beethoven* (*btv*), and *touch-insensitive-larva-B* (*tilB*)[45]), and scholars know little of how these mutations might affect song amplitude. In the three paragraphs below we attempt to integrate current knowledge on song amplitude generation with knowledge of mutations that influence song amplitude.

The auditory mutants *ato*, *btv*, and *tilB* have impaired hearing and Tauber and colleagues suggest that these mutants may sing louder because they lack auditory feedback.[45] However the ability to hear is not necessary for AMD which mainly is controlled by visual signals,[31] a finding that may question the importance of auditory feedback in amplitude control. Note that *ato*, *btv*, and *tilB* are pleiotropic and likely affect not only audition but other functions that may help explain why these mutants sing louder than controls.[45]

The Calcium channel mutant *cacaphony* (*cac*) sings a distorted song which is larger in amplitude and which has pulses with more cycles than normally found in wild type male *D. melanogaster*. *Cacaphony* encodes a subunit of voltage activated Calcium channels (primary a1 subunit)[57] and is involved in neurotransmitter release at the presynaptic terminus of the neuromuscular junction.[61] As song amplitude is linked to

muscle activity and muscle power,[31,62] *cac* action at the neuromuscular junction might explain why *cac* mutants song amplitude is distorted. In addition *cac* mutants show subtle visual defects,[57] which is interesting as the male controls amplitude partially by visually estimating his distance to a female.[31] Thus the fact that *cac* mutants sing at increased amplitude could be due to the neuromuscular effect of *cac*, the visual defect of *cac* mutants, or both. Note that voltage-gated calcium channels are expressed in many tissues throughout development, and it is therefore difficult to interpret the mechanism leading to distorted song in the pleiotropic mutant *cacophony*.

*Dissonance* (*diss*) is an allele of the *non-on-transient-A* (*nonA*) locus in *Drosophila* and is protein-coding, potentially for an RNA-binding protein.[63] Mutants of *diss* sing an abnormal courtship song; their song bouts start normal, but progressively deviate to become abnormally loud and polycyclic, which suggests a role of *diss* in song amplitude control. Interestingly *diss* mutants exhibit optomotor deficiencies during courtship, reportedly 'courting nothing',[60,63] which agrees with the reported role of vision in amplitude control.[31] Additionally *diss* is expressed in thoracic muscles,[63] which is in agreement with the reported role of thoracic muscles in amplitude control.[31,43] Note, however, that *diss* is expressed in many other tissues, including neuronal and non-neuronal cells throughout development,[63] which suggests that the song amplitude phenotype of *diss* mutants may be explained by factors other than vision and thoracic muscle function. To study the mechanism of how genes like *ato*, *btv*, *tilB*, *cac*, and *diss*, affect song amplitude, it is necessary to generate transgenic constructs that allow targeted control of gene expression throughout development and in different tissues.

## 1.7 How do fruit flies hear?

The fly ear includes the arista, the funiculus and the pedical (second antennal segment, see Figure 1.2a). Fruit flies hear with arista, which is a feathery structure on their antennae (Figure 1.2a). When a fruit fly is stimulated acoustically, the arista vibrates together with the funiculus (third antennal segments), which is attached to the arista (Figure 1.2a and 1.2b). The female arista is tuned to the song frequency of males from their species[15] and female flies copulate more when they hear courtship song with their species frequency,[36] which suggests that the female ear evolved to discriminate fly song of different species.

The funiculus attaches to the pedical which harbours about 480 Johnston Organ (JO) neurons[64]—the primary auditory neurons in *Drosophila*. The JO neurons are distributed over the inner surface of the pedical and attach to the pedical–funiculus joint with elongated cilia that are stretched when the funiculus vibrates, thus activating the stretch-activated JO neurons (Figure 1.2b). JO neurons are necessary for hearing in flies: when JO neurons are silenced, flies are rendered deaf and do not exhibit a behavioural response to song playback.[65]

Arista may be involved in the processing of song amplitude: arista are displaced by air particles[15] and fast particles may displace the arista more than slow particles. Thus the displacement of the arista depends on air particle velocity, which depends on song amplitude.[66] The fly ear actively amplifies arista displacement through active amplification—a process in which JO neurons, that are motile mechanosensory cells, contribute to the vibrations of the arista and the funiculus.[67,68] Active amplification intensifies low-amplitude stimuli relative to high-amplitude stimuli[67] and thus attenuates differences in song amplitude.

JO neurons project to the antennal mechanosensory and motor center (AMMC).[69–72] There is also anatomical evidence that JO neurons directly project to olfactory and visual centers, and to the thoracico-abdominal ganglia,[69] but these connections have not yet been functionally studied. Two classes of AMMC neurons, the projection neuron aPN1 and the lateral interneuron aLN are necessary for female responses to song.[70] aPN1 integrate pulse rate in female[70] and male[72] *Drosophila*, suggesting a role of aPN1 in courtship song processing.

aPN1 projections overlap with the male-specific ventrolateral protocerebrum projection neurons 1 (vPN1),[72] which suggests that auditory information is transmitted from aPN1 to vPN1 in males. And the vPN1 are indeed involved in the processing of courtship song: they are necessary for male responses to song[72] and seem involved in the processing of inter-pulse intervals (IPIs) in courtship song. vPN1 is anatomically connected with high-order integration neurons in the protocerebrum (pC1) in males.[72] pC1 neurons are necessary for male and female responses to song[71,72] and are involved in IPI processing in males,[72] which suggests a role of pC1 neurons in the species-specific response of flies to IPI.[29,66,72] See Figure 1.2c for a simplified model schematic of the auditory circuit in *D. melanogaster*. See[69] for a comprehensive map of secondary auditory interneurons, including AMMC, aPN1 and aLN.

## 1.8 Thesis outline

In Chapter 2 we designed a mathematical model of *Drosophila* courtship song production in which we assume that neuromuscular dynamics shape pulse song amplitude. We discuss studies on the neuronal, behavioural and muscular basis of courtship song production and then formulate our model based on these studies.

Based on our neuromuscular model we define in Chapter 3 the novel song parameter song amplitude structure (SAS). We then discuss the relationship of AMD and SAS. Recently, Coen et al.[31] reported that male flies actively modulate their song amplitude relative to distance from females: males sing louder when a female is farther away, suggesting AMD in *Drosophila*. In the same paper, they also noted that vision cannot fully explain amplitude modulation, as there are several additional sources of amplitude variation (e.g., changing orientation of a male's wing relative to a microphone,[43] physical limitations, wing size, and male speed[31]). We suggest that neuromuscular dynamics can help explain amplitude variations that cannot be explained by distance, and we suggest to quantify amplitude variations in *Drosophila* song with SAS. Our neuromuscular model predicts SAS to signal muscle power, and we validate the predictive power of our model using muscle mutants.[74]

In Chapter 4 we demonstrate that SAS is a communication signal in the fruit fly *Drosophila*. Our work complements the recent finding that male flies actively control song amplitude by demonstrating that receivers can decode messages in the amplitude structure of fly song. We show that SAS differs in the closely related species *D. melanogaster* and *D. simulans* and that females prefer their own species' SAS and can differentiate between different SASs, which suggests that SAS is a communication signal in *Drosophila*. We also show that male *D. melanogaster* and male *D. simulans* differentiate SASs, which suggests that both *Drosophila* males and females can process SAS.

Perceived amplitude is subject to noise[4,31,44,75] and thus responses to SAS may be affected by noise. In Chapter 5 we investigate whether noise in SAS affects behavioural responses. For this we generate artificial song playbacks and mimic noise by randomising the SAS signal and ask whether flies respond differently to song with random versus real SAS. We find that *D. melanogaster* females copulate at similar rates to both types of song, which suggests that females are resilient to noise in the SAS signal. Conversely, we find that male *D. melanogaster* respond more to real than to random SAS, suggesting that randomisation disrupts the male response to the SAS signal. Our findings suggest that female and male flies vary in their tolerance

to randomised SAS. We discuss our results in the context of Chapter 4 in which we see that females and males respond in a similar manner to SAS modulations. We suggest randomised SAS mimics noise in SAS and females and males differ in their response to this noise and we speculate on an evolutionary explanation for sex differences in response to SAS noise.

In Chapter 6 we further investigate the male response to randomised SAS. Males respond less to randomised SAS than to real SAS, which suggests males can differentiate between real and random SAS. We study male response to random SAS and show that males can be resilient to short pulse trains with random SAS that alternate with pulse trains of real SAS (less than about one second long), whereas playbacks with longer pulse trains of random SAS do not stimulate male auditory responses. We speculate that the behavioural resilience to short pulse trains with random SAS that we observe in male flies may be related to auditory restoration, which describes the restoration of short periods of missing information in sounds,[76] and we compare and contrast the phenomenon we observe with auditory restoration in mammals, rodents and birds.[77–83]

In Chapter 7 we raise questions that arise from our work and discuss potential ways to study these questions. SAS is a communication signal and fly movement may affect received song amplitude[31] so we discuss the question 'How does fly movement affect received song amplitude?'. This question has not yet been studied, but we suggest that it is possible to study this question by combining established experimental set-ups.[31,47] We then consider the question 'How could flies auditory process SAS?' and suggest that flies may spectrally decompose song[64] to extract information on SAS, or bandpass-filter song[84] to extract information on SAS, or both. In our mathematical model of courtship song production we assume that SAS reflects neuromuscular dynamics. We point out that a recently published experimental method for imaging muscle activity in alive flies[20] may allow our model assumption to be tested. We end by pointing out that SAS may be a species-specific communication signal in *Drosophila*, and we acknowledge that additional fly species should be tested to affirm that SAS is a species-specific communication signal. What species should be tested and why? We suggest criteria that may help to choose additional species for studying SAS.

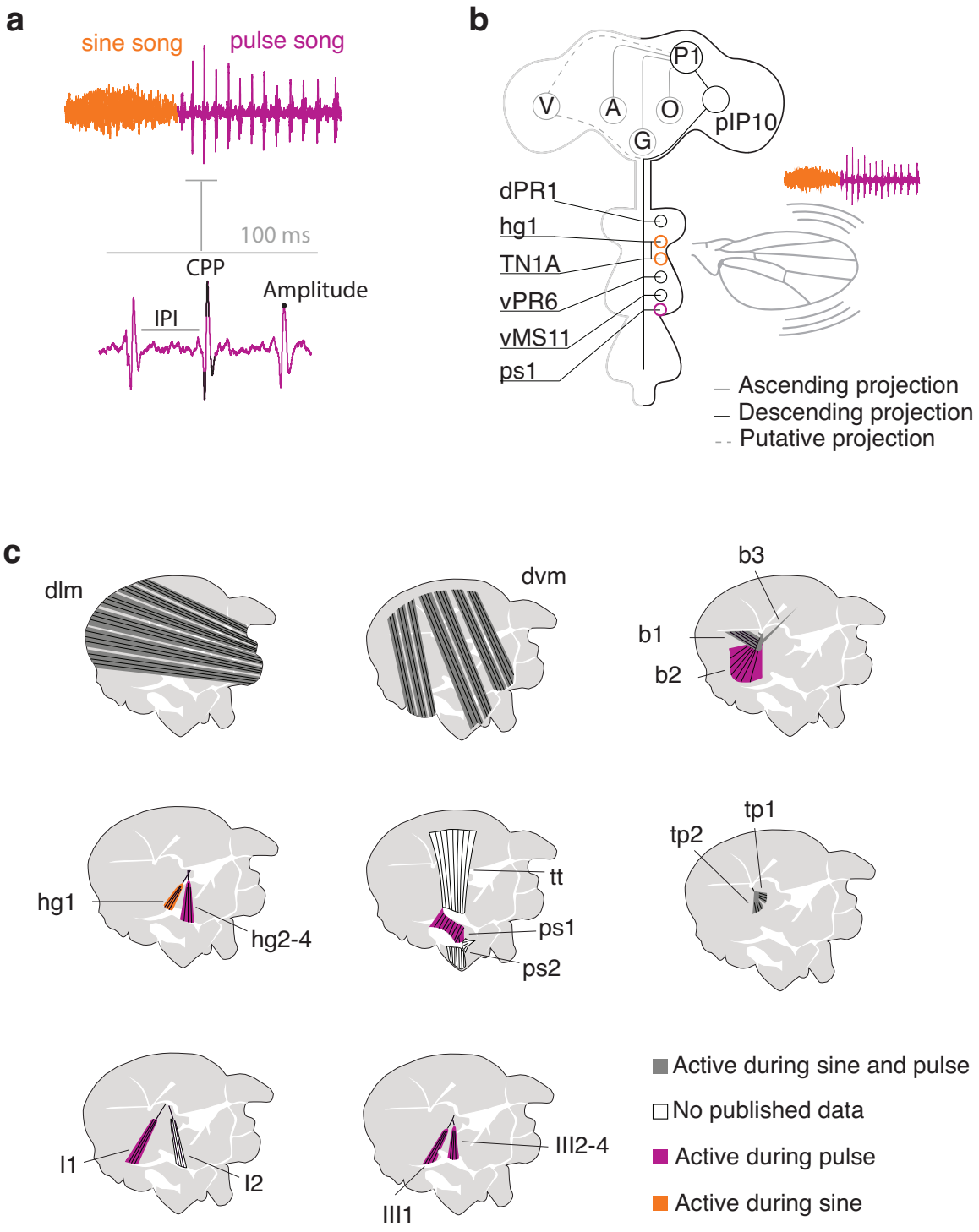



**Figure 1.1:** Neural and muscular control of sine and pulse song in *Drosophila melanogaster*. (**a**) Sample trace of courtship song of a *D. melanogaster* male. The lower song trace magnifies a section of the upper song trace. We illustrate pulse song parameters [inter-pulse interval (IPI), cycles per pulse (CPP), and amplitude] in the lower song trace. (**b**) Schematic of proposed neural circuitry controlling courtship song.[21] Wing extension and song are under multisensory control: vision (V), audition (A), gustation (G) and olfaction (O) affect wing extension or modulate song. We colour-code neural clusters that affect sine with orange circles, neural clusters that affect pulse song with purple circles and neural clusters that may affect both types of song with black circles. (**c**) Schematics of muscles involved in wing extension from Dickinson et al.[53] with added colour-coding.

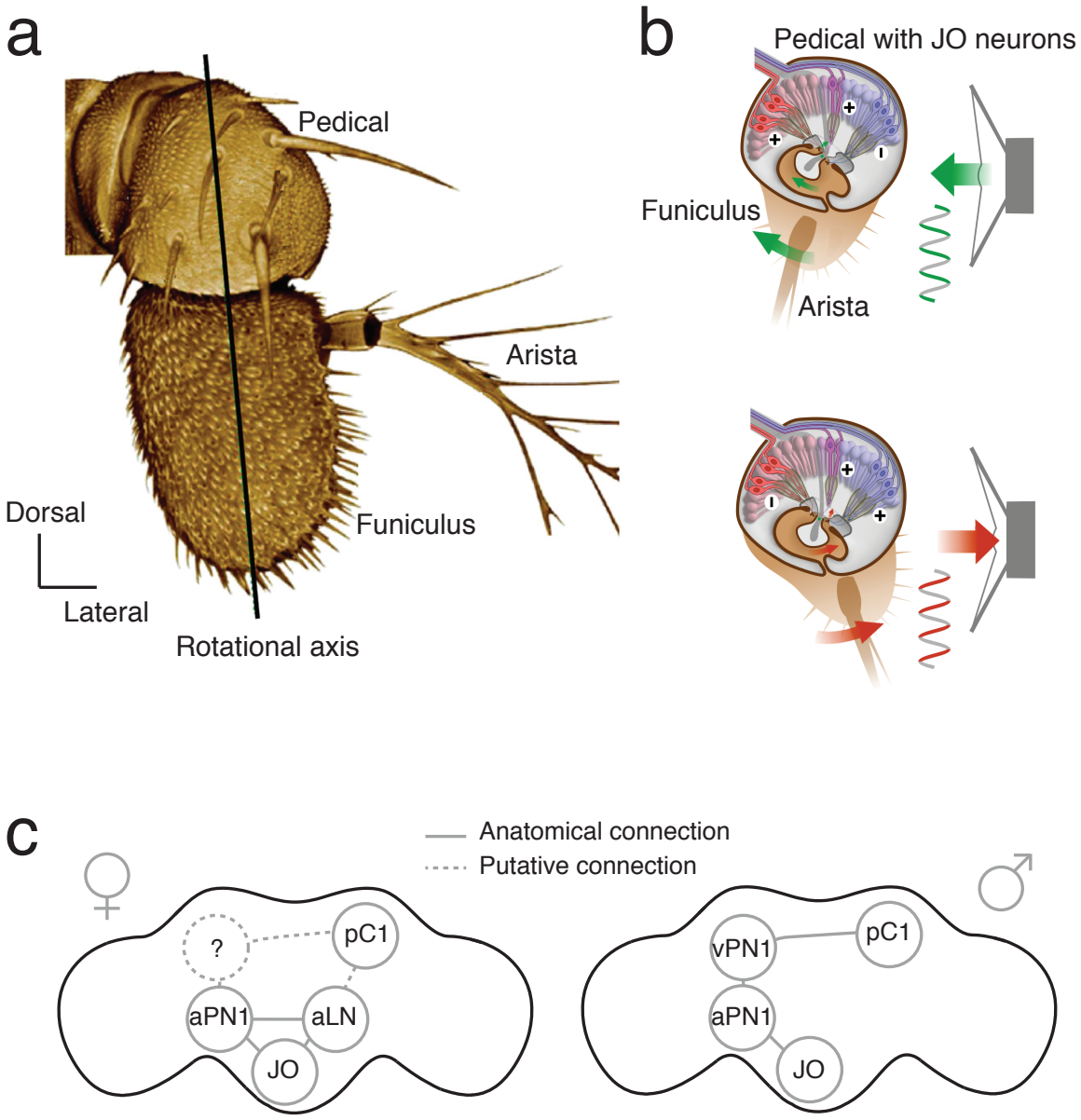



**Figure 1.2:** The auditory system of *Drosophila melanogaster*.
(**a**) The fly ear includes the arista, the funiculus (third antennal segment), and the pedical (second antennal segment). We indicate the rotational axis of funiculus as black line. (**b**) The pedical contains Johnston Organs (JO), which are the primary auditory neurons in *Drosophila*. JO neurons are aligned along the inner surface of the pedical and have extended cilia that connect them to the pedical–funiculus joint. With acoustic stimulation the funiculus and the arista vibrate back (green, on positive pressure) and forward (red, on negative pressure). The vibration can depolarize (+) or hyperpolarize (−) JO depending on the movement of the arista and the position of JO. Subpanel (a) and (b) are adapted from.[73] (**c**) Simplified schematic of auditory circuitry in female and male flies. In female flies JO neurons connect with projection neurons (aPN1) and local interneurons (aLN) of the antennal mechanosensory and motor center (AMMC). These secondary auditory neurons may connect to the processing centre neurons in the protocerebrum (pC1). (The question mark flags the site of vPN1, which are male-specific and thus absent in females.) In male flies JO connect to aPN1 neurons, which connect to vPN1, which connect to pC1 neurons.

# Chapter 2

# How do fruitflies sing? Models of courtship song production

## 2.1 Introduction

Many models of courtship song production in *Drosophila* exist, focusing on neuronal control,[30,31,37,38,85,86] behavioural observations[31,40] or the muscular basis of song production.[48,51] However some of the existing models of courtship song production are mutually contradictory[37,85] and no unifying account exists so far. Therefore we discuss models of song production in Sections 2.1.1, 2.1.2 and and suggest a unifying model in Section 2.2. Note that so far no mathematical formulation of fly song exists in the literature, and we formalise our model mathematically. A mathematical formalisation can be helpful as it explicitly states assumptions which other models may not.[30,85] In addition we derive a computational simulation from our model, which we use to derive predictions about fly song. Importantly our model motivates the experimental work we discuss in Chapters 3, 4, 5, and 6. In addition our model creates the hypothesis for the rest of the thesis, namely that song amplitude structure (SAS) is important and may act as communication signal.

### 2.1.1 Neuronal and muscular control of song

Courtship song is under multisensory control and sensitive to visual,[31,40] auditory,[70,72] olfactory and gustatory stimulation[46,47,87,88] (Figure 1.1a). Courtship song can be triggered by touching a tethered male with the abdomen of a female,[47,88] making him taste the female through gustatory neurons.[46,89] Smell can also influence courtship

song initiation: The male-specific volatile pheromone 11-cis-vaccenyl acetate (cVA) can inhibit singing in males.[47] Sensory stimuli are integrated in the higher-order processing centre P1,[47,71] which is thought to act as a command cluster for courtship initiation.[21] pC1 neurons, which comprise some P1 neurons, are activated in response to courtship song playbacks,[71,72] demonstrating a role of pC1 in integrating auditory information. Interestingly, activation of pC1 neurons in males is sufficient to initiate courtship song production[30,72] (Figure 2.1a), highlighting the importance of these neurons in both sensory integration and motor activation. Males also use visual stimuli to modulate their courtship song:[31,40] Males can adjust the intensity of their courtship song and sing louder when more distant to the female.[31] We propose a model in which central P1 neurons act as integrators of visual,[31,40] auditory,[70,72] olfactory[47] and gustatory information,[46,89] and in addition trigger the motor programme for courtship song production[30,72] (Figure 1.1b).

### 2.1.2 Switching between sine and pulse song

Clyne and Miesenböck showed that headless artificially excited flies can produce courtship song, demonstrating that thoracic pattern generators are sufficient for producing courtship song that is similar to song of intact males[85] (see Figure 2.1b). Indeed the authors observed that the song of these headless flies included switches between sine and pulse song, raising the question of how switches between these two song modes are controlled. Clyne and Miesenböck suggest two alternative models to explain switches between sine and pulse song. In their first model, song is controlled by one pattern generator, that acts as an on-off switch for song. Switches between sine and pulse are controlled by a second switch, that sets the mode of song.[85] This model suggests that one set of neurons controls both sine and pulse song. In contrast, Shirangi et al. show that sine and pulse song are controlled by different neurons: they identified a single motor neuron, hg1, that, when switched off, eradicates sine without affecting pulse song[37] (see Figure 2.1b). Shirangi et al. also identified an interneuron TNA1, that when switched off eradicates sine song.[38] This demonstrates that sine and pulse song are controlled by independent circuits, which is in contrast with the one pattern generator model suggested by Clyne and Miesenböck.[85] In the alternative model Clyne and Miesenböck suggested switches between sine and pulse song result from mutual inhibition of a sine and a pulse pattern generator.[85] This model is in agreement with the finding of Shirangi et al. that independent neurons control sine and pulse song. However motor units controlling sine song are active at the same

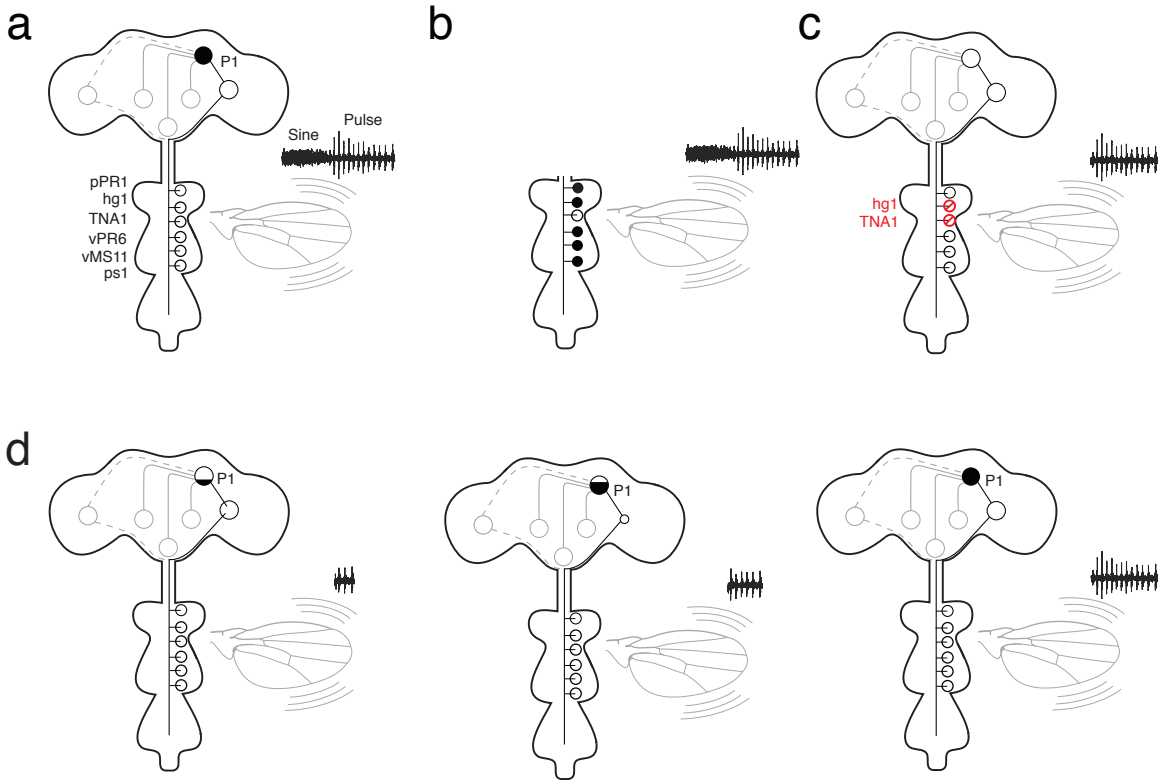



**Figure 2.1:** Artificial activation of song control neurons and its effect on courtship song.
(**a**) When P1 command neurons in the brain of male flies are artificially activated they sing naturalistic courtship song in the absence of females,[30] which suggests that P1 neuron activity is sufficient to drive naturalistic singing. We indicate artificial activation with black circles, white circles are not artificially activated. (**b**) Decapitated flies sing naturalistic courtship song when a set of neurons in the ventral nerve court is activated.[85] This suggests that activation of neurons in the ventral nerve court is sufficient to drive naturalistic singing. Note that Clyne and Miesenböck activated additional neuronal clusters to the ones we indicate. We show only neuronal clusters that are known to influence courtship song.[30] (**c**) When TNA1 neurons are silenced male flies do not sing sine song but they sing only pulse song,[38] which suggests that TNA1 neurons are necessary for singing sine. Similarly, when the hg1 motorneuron is silenced flies sing only pulse and no sine song,[37] which suggests that hg1 and TNA1 neurons may make up a sine song pattern generator. We indicate silencing by red circles with red diagonals. (**d**) Pulse song rate depends on the intensity of neural activation of P1 command neurons: the more P1 is artificially activated, the more pulse song flies sing.[30]

time as motor units controlling pulse song[48,51] (Figure 1.1b), which suggests that sine and pulse central pattern generator (CPG) are active at the same time, and may not inhibit one another as suggested.[85,86] Importantly the firing pattern of most sine motor neurons does not change after a switch from sine to pulse song,[48] which implies that pulse song does not affect the activity of most sine motor neurons. However the firing frequency of basalar muscles b1 and b3 (Figure 1.1b) is smaller when a fly sings pulse song than when a fly sings sine song, which suggests that these basalar muscles may be inhibited during pulse. Hence physiological data partially support the notion

that there may be some inhibition between CPG that control sine and pulse.

Pulse production requires higher level of excitement than sine production.[30,32,39,40] Von Philipsborn et al. showed that increasing excitement makes males sing more pulse song by artificially activating the neuronal cluster P1[30] (Figure 2.1d-f), which demonstrates that neuronal activity promotes pulse song production. Behavioural studies in wild-type males imply that excitement drives switches between sine and pulse.[39,40] Trott et al. find that the locomotor activity of the courting fly couple correlates with the amount of pulse: the faster the couple moves the more pulse the male sings.[39] Coen et al. show that males increase their speed before switching to sing pulse.[40] Conversely, before switching from pulse to sine, males decrease their speed, which implies that changes in speed can predict decisions for singing sine or pulse.[40] Why is speed linked to decisions what type of song to sing? Animals increase their speed when they are excited and speed may indicate excitement. Theories that explain decision-making with excitement states have a long tradition that reaches back to Lorenz and Tinbergen[90,91] and are applied to date, for example in modelling grooming in *Drosophila melanogaster*[92] and in modelling transitions between swimming and walking in salamander.[93] In our model we assume that excitement affects decisions for singing in *Drosophila*. Further we assume that pulse production requires higher level of excitement than sine production[30,39,40] and we model excitement as frequency of neuronal firing.

One way to model excitatory hierarchy is by setting neuronal activity thresholds: When neuronal activity is low, a fly would sing sine, when neuronal activity is high, a fly would sing pulse. If true, one would expect—at low neuronal activity levels—flies to sing sine only. But this is not the case as flies sing both sine and pulse when neuronal activity is low.[30,85] This implies that the neuronal thresholds may not be appropriate for modelling an excitatory hierarchy in song. What would be an alternative to neuronal thresholds? When flies sing pulse song more of their muscles are active then when flies sing sine.[48,51] (See Figure 1.1 for an illustration of muscles active during sine singing and pulse singing.) This suggests that activity of muscles may be linked to what type of song a fly sings. We assume muscle activity thresholds in our model: When muscle activity is low, a fly sings sine, when muscle activity is high, a fly sings pulse.

## 2.2 Mathematical model of fly song and model predictions

We decided to mathematically model courtship song and to make explicit assumptions that are experimentally testable. Figure 2.2 depicts a chart of model structure. Our model is based on three main assumptions:

1. Two song CPG exist, one for sine and one for pulse song generation.[37,38,48] The two song CPG are active simultaneously.[48,85,86] For simplicity we assume no interaction between sine and pulse CPG.

2. Separate muscles for sine and pulse production exist.[37,48]

3. Song features as amplitude, frequency and inter-pulse-interval pattern[1] are controlled by muscle activity.[48,94,95] We assume muscle activity and song feature intensity correlate positively.[31,43]

In Figure 2.2 each model symbol can stand for a set of entities. For example the sine muscle triangle stands for the large dorsal muscles, and the basalar muscles b1 and b3, which are active during sine song production[48] and the hg1 muscle that is necessary for sine song.[37] The pulse model muscle, that controls amplitude and frequency of pulse song, stands for the basalar muscle b2[48] and the ps1 muscle.[37] The second pulse model muscle that controls pattern of pulse, stands for the five axillary muscles (I1, III2-4), that according to Bennet-Clark and Ewing are sufficient to generate the pattern of alternating high amplitude pulses and low amplitude inter pulse intervals[48,94] (Figure 1.1b). Note that this assignment of real muscles to model muscles is a working hypothesis and based on recordings of nine of 20 thoracic muscles potentially involved in courtship song production[48,53] and additionally on silencing of two of these muscles, hg1 and ps1.[37]

Our model command CPG encompasses around 40 P1 neurons and two pIP10 neurons[30] (Figure 1.1a), which mediate the decision to sing and trigger realistic song production.[30] Two motor neurons have been described that specifically affect sine respectively pulse song.[37] The hg1 neuron is part of the sine CPG, while the ps1 neuron is part of the pulse CPG.[37] In addition the TNA1 thoracic circuit connects with the hg1 neuron and is also necessary for sine song production.[38] Candidate neurons of sine and pulse CPG encompass at least three classes of neurons (dPR1,

[1] According to Ewing the activity of axillary muscles is sufficient to generate alternating high amplitude peaks and low amplitude IPIs. In accordance with Ewing we choose to implement inter pulse interval (IPI) pattern with a neuromuscular mechanism.[48]

vPR6 and vMS11).[30] The pulse pattern CPG include vPR6 neurons which control the duration of the inter-pulse interval (IPI)[30] and vMS11 neurons which control the cycles per pulse.[30] For assigning additional neurons to the sine respectively the pulse CPG, one will need to asses how neurons are connected with sine and pulse muscles and motor neurons as hg1 and ps1.

### 2.2.1 Model Specification

Our song models consists of three components: neurons, muscles and song. Below we specify essentials of each of the components, while we give additional information in the Appendix. We have implemented the model equations in MATLAB.[96]

#### 2.2.1.1 Neurons

We model incidence of neuronal Action Potentials (AP) as random, as no specific pattern of activation is necessary for realistic courtship song production.[30,85,86] Note that we simplify neuronal activity to binary on-off states and thus focus on the frequency of AP. Frequency of AP represent neuronal excitement levels that are important for song choice[30] (see Figure 2.1d-f).

The command CPG $\Omega$ yields input $s$ to the sine pattern generator, such that

$$s(t) = \Omega(t). \tag{2.1}$$

With this the excitation pattern of $\Omega$ and $s$ are equal. Thus one of these functions is redundant. However we chose to include both the command CPG $\Omega$ and the sine CPG $s$, as this reflects the physiological structure of the song circuit.[30,37,38,51]

The command neuron $\Omega$ yields input to the pulse pattern generator $p$ with a time lag constant $a$, $a \in \mathbb{Z}^+$., such that

$$p(t) = \begin{cases} 0, & \text{if } 0 \leq t \leq a \\ \Omega(t-a), & t > a. \end{cases} \tag{2.2}$$

Thus activity in the pulse CPG is equivalent to activity in sine CPG apart from a temporal lag determined by constant $a$. Constant $a$ represents possible differences in conduction speed from the command CPG to the lower level CPG.

The third CPG in our model is the pattern neuron $p_{\text{pap}}(t)$ that controls alternations between high amplitude peaks and low amplitude inter-pulse intervals and the cycles per pulse.[48] Activity of the pulse pattern CPG is controlled by muscle tension level of the pulse frequency-amplitude muscle. Only when muscle tension exceeds threshold $m$ the pulse pattern CPG is activated. The function of activity of the pulse amplitude pattern CPG, $p_{\text{pap}}(t)$, is defined in the Appendix B, as the pulse pattern CPG is not essential for song choice but patterns pulse only.

The pulse amplitude pattern neuron $p_{\text{pap}}$ becomes active when the contraction level of the pulse amplitude and frequency muscle $x_{\text{paf}}$ exceeds threshold constant $m$, so that

$$h_1(t) = \begin{cases} 1, & \text{if } x_{\text{paf}}(t) \geq m \\ 0, & \text{otherwise.} \end{cases} \tag{2.3}$$

We assume $p_{\text{pap}}$ to fire in invariant intervals $Z$, thus we require time bins indexed in $h_1$ to have a minimal duration of $Z$, which gives the second helper function $h_2(t)$ :

$$h_2(t) = \begin{cases} h_1(t), & \text{if } \exists\ t \in Q \text{ such that } h_1(t) = 1 \\ 0, & \text{otherwise} \end{cases} \tag{2.4}$$

with $Q := [t - Z - 1,\ t - 1]$. Figure 2.3 illustrates helper functions $h_1(t)$ and $h_2(t)$.

In time intervals $Z$ we define an excitatory pattern $p_{\text{papunit}}$ that represents an AP followed by a refractory period such that

$$p_{\text{papunit}} = \begin{cases} 1, & \text{for } 0 \leq t \leq z \\ 0, & \text{for } z < t < Z \end{cases} \tag{2.5}$$

with duration $z$ of one AP $z \in \mathbb{R}^+$ and duration of one AP and it's refraction time $Z \in \mathbb{Z}_0^+$ and $Z > z$ for all $z$.

We expand the excitatory pattern $p_{\text{papunit}}$ to indices determined by helper $h_2$ to receive $h_{\text{pat}}(t)$:

$$h_{\text{pat}}(t) = \begin{cases} 1, & \text{if } h_2(t) = 1 \\ 2, & \text{if } h_2(t+1) = 1 \\ \ldots & \\ Z, & \text{if } h_2(t+Z) = 1 \\ 0, & \text{otherwise.} \end{cases} \tag{2.6}$$

The final activity pattern of neuron $p_{\text{pap}}$ is then given by

$$p_{\text{pap}}(t) = p_{\text{papunit}}(h_{\text{pat}}(t)). \tag{2.7}$$

#### 2.2.1.2 Muscles

We model muscle contraction dynamics with a generic muscle function $x_i(t)$, simplified from Brezina et al.,[97] such that

$$x_i(t) = \frac{1}{1 + e^{-k_i(f_i(t) - g_i)}}. \tag{2.8}$$

where $x_i(0) = 0$ and $x_i(t) \in [0, 1]$. The generic muscle function $x_i(t)$ takes the form of a sigmoid with constants $k_i$ and $g_i$. Constant $k_i$ determines the slope of muscle contraction, and $g_i$ sets the offset of the sigmoid and in effect determines the immediacy of muscle contraction on neuronal excitation. The muscle contracts when

it receives an AP from neuron $y_i$ such that

$$f_i(t+1) = \begin{cases} f_i(t) + 1, & \text{if } y_i(t) = 1 \\ f_i(t) - d, & \text{otherwise.} \end{cases} \tag{2.9}$$

where $f_i(0) = 0$ and $f_i(t) \in [0, n]$ and the decay constant $d \in [0, 1]$. Constant $n$ is the upper boundary of function $f_i$. Because boundary $n$ and offset $g_i$ are discrete, $n, g_i \in \mathbb{N}_0^+$, while $j_i, k_i \in \mathbb{R}_0^+$.

The generic neuron function $y_i$ represents input of model neurons $s(t)$, $p(t)$ or $p_{\text{pap}}(t)$ with the following connections between neurons and muscles:

I. Sine amplitude and frequency muscle with $i = \text{saf}$, $y_i(t) = s(t)$.

II. Pulse amplitude and frequency muscle with $i = \text{paf}$, $y_i(t) = p(t)$.

III. Pulse amplitude patterning muscle with $i = \text{pap}$, $y_i(t) = p_{\text{pap}}(t)$.

Because the decay constant $d$ lies between 0 and 1 muscles can relax slower than they contract. Therefore neuronal firing frequency can induce accumulating muscle tension. When the magnitude of function $f_i(t)$ increases, $x_i(t)$ increases sigmoidally.

Neuronal input to sine and pulse muscles is similar, however integrated differently by the two muscles: the sine muscle has a steeper contraction slope than the pulse muscle such that $x_{\text{saf}} > x_{\text{paf}}$. Thus the same amount of continuous excitation can induce full contraction in the sine, but only partial contraction in the pulse muscle, which we illustrate in Figure 2.5. Note that we show a fourth model muscle in Figure 2.5, $x_{\text{psaf}}$ which is helpful for mapping actual muscles to our model muscles. The model muscle $x_{\text{psaf}}$ is not part of our MATLAB implementation.

#### 2.2.1.3 Song

Function $v_{\text{f}}$ computes song frequency by averaging over frequencies controlled by sine and pulse muscles. The tension of these muscles is converted to song frequency by multiplying muscle tension $x_{\text{saf}}$ and $x_{\text{paf}}$ with constants $j_{\text{saf}}$ and $j_{\text{paf}}$ respectively. Song

frequency $v_{\mathrm{f}}$ is computed over successive song wave periods $\tau_i$:

$$v_{\mathrm{f}}(t) = \begin{cases} 1, & \text{for } t = 0 \\ \frac{1}{\#\tau_i} \sum_{z \in \tau_i} \Big( j_{\mathrm{saf}} \times x_{\mathrm{saf}}(z) + j_{\mathrm{paf}} \times x_{\mathrm{paf}}(z) \Big) & \text{otherwise,} \end{cases} \tag{2.10}$$

where $z$ denotes to time samples in song wave $\tau_i$ and where $\#\tau_i$ is the cardinality of $\tau_i$. The duration of song wave $\tau_i$ depends on average song frequency during a preceding song wave such that:

$$\tau_i = \begin{cases} \mathrm{mean}(v_f(z)) & z \in [1, Fs \times 0.05] \text{ for } i = 1 \\ \mathrm{mean}(v_f(z)) & \text{for } z \in \tau_{i-1} \text{ for all } i > 1 \end{cases} \tag{2.11}$$

where we use MATLAB notation for computing mean frequency. We define the first song wave $\tau_1$ to encompass the first 50 milliseconds of a song simulation as this is the average duration of song waves in courtship song of *D. melanogaster*.[32] We multiply sampling frequency $Fs$ with 0.05 seconds to transform time to time samples $t$. The time scale $t$ of our model is a count of discrete fractions of a second, with $t \in \mathbb{N}_0^+$. The number of fractions is determined by the sampling frequency $Fs$, with $Fs \in \mathbb{N}_1^+$.

Calculation of average amplitude $v_{\mathrm{a}}$ per song wave $\tau_i$ corresponds to average frequency calculation in Equation 2.10. The only difference is that conversion rates from muscle tension to amplitude are 1, as we assume muscle tension and amplitude to be normalised between 0 and 1. This simplifies Equation 2.10 to:

$$v_{\mathrm{a}}(t) = \begin{cases} 0, & \text{for } t = 0 \\ \frac{1}{\#\tau_i} \sum_{z \in \tau_i} \Big( x_{\mathrm{saf}}(z) + x_{\mathrm{paf}}(z) \Big) & \text{otherwise,} \end{cases} \tag{2.12}$$

We base both song frequency $v_{\mathrm{f}}$ and song amplitude $v_{\mathrm{a}}$ on the same set of muscles to reduce model complexity.

Patterned amplitude of pulse song for alterations between pulse peaks and IPI

$v_{\text{pap}}$ is assessed similarly to frequency and amplitude:

$$v_{\text{pap}}(t) = \begin{cases} 0, & \text{for } t = 0 \\ \frac{1}{\#\tau_i} \sum_{z \in \tau_i} x_{\text{pap}}(z) & \text{otherwise.} \end{cases} \tag{2.13}$$

In contrast to frequency and amplitude, patterned amplitude of pulse is controlled by one model muscle only.

To apply frequency function $v_{\text{f}}$ on the MATLAB sin function, we introduce helper function $u$ that enumerates time samples $z$ of each song wave period $\tau_i$. Then we modulate the carrier sine wave sin with frequency $v_{\text{f}}$, amplitude $v_{\text{a}}$ and pattern $v_{\text{pap}}$ (see Figure 2.4) such that

$$\text{𝄞}(\text{t}) = \Big(v_{\text{pap}}(t) + \alpha\Big)\ v_{\text{a}}(t)\ \sin\Big(v_{\text{f}}(t)\ u(t)\Big). \tag{2.14}$$

Constant $\alpha$, with $\alpha \in [0, 1]$, is the upper amplitude boundary of sine song and IPI amplitude.

We visualise the transformation from neuronal activity, to muscular tension and then to song in Figure 2.5.

#### 2.2.1.4 Model Parameters

We list our model's parameters in Appendix B, Table A.1 and we visualise how parameters can affect courtship song in Figure 2.6.

### 2.2.2 Model of neuromuscular control of song amplitude

Here we present a special case of our model that is helpful for explaining song amplitude structure (SAS), which are characteristic patterns in song amplitude (see Figure 2.7).

We assume that SAS reflects neuromuscular dynamics. It is known that song amplitude in birds correlates positively with electromyographic (EMG) recordings of vocalizing muscles,[14] and assuming a linear relationship between song amplitude and muscle activity is the simplest way to model such a relationship, which is why we assume this for our model.

A song with an SAS of $S_{\mathrm{SAS}}$ is a product of a constant-amplitude song $S_c$ and a composite function of muscle activity $m_a$ and neuronal activity $\tilde{n}_a$. We thus write

$$S_{\mathrm{SAS}}(t) = S_c(t) \times m_a(\tilde{n}_a(t))\,, \tag{2.15}$$

where $t$ is discrete time. Note that only a subset of neurons and muscles can influence song amplitude,[31,37] and we consider only this subset in our model. We specify the neuronal activity

$$\tilde{n}_a(t) = \begin{cases} 1\,, & \text{if neurons are active}\,, \\ \\ 0\,, & \text{if neurons are inactive}\,. \end{cases} \tag{2.16}$$

We model muscle activity as a sigmoidal function:[97]

$$m_a(t) = \begin{cases} \frac{1}{1+e^{-k_i(f(t)-g)}}\,, & \text{if } \tilde{n}_a(t-1) = 1\,, \\ \\ \frac{1}{1+e^{r\times k_i(f(t)-g)}}\,, & \text{if } \tilde{n}_a(t-1) = 0\,, \end{cases} \tag{2.17}$$

where $r$ is a muscle-relaxation constant, $k$ is a gain constant, and $g$ is the muscle-contraction delay. (See table 2.1 for a detailed description of $r$, $k$ and $g$.)

| Model parameter | Description |
|---|---|
| $r$ | The relaxation constant $r$ determines how fast a muscle relaxes relative to how fast it contracts. We generate Figure 2.7a using $r = 0.5$, which makes a muscle relax half as fast as it contracts and reflects physiological properties of muscles.[97,98] |
| $k$ | The gain constant $k$ determines how fast a muscle contracts. In the left panels of Figures 2.7a, we set $k_1 = 0.25$, which gives a contraction time that reflects the contraction times of insect muscles at tetanic stimulation.[98] To illustrate slower muscle contraction, we also consider smaller values of $k$. We use $k_2 = 0.5 \times k_1$ for the middle panels of Figures 2.7a, and we use $k_3 = 0.25 \times k_1$ for the right panels of Figures 2.7a. |
| $g$ | The muscle-contraction delay $g$ determines when a muscle starts contracting. To start contraction at $t = 0$, we use a delay of $g_1 = 25$ for gain $k_1$, a delay of $g_2 = 50$ for gain $k_2$, and a delay of $g_3 = 100$ for gain $k_3$. |

**Table 2.1:** Neuromuscular model parameters and parameter description.

To obtain Figure 2.7a, we set $m_a(0) = 0$ and define $f(t)$ such that

$$f(t) = \begin{cases} t\,, & \text{for } t \in \{0, \ldots, 50\} \quad \text{(the muscle is activated)}\,, \\ t - 50\,, & \text{for } t \in \{51, \ldots, 150\} \quad \text{(the muscle is not activated)}\,. \end{cases} \tag{2.18}$$

We then simulate muscle activity $m_a(t)$ with the parameters in Table 2.1.

We choose the parameter values in Table 2.1 because they approximate experimentally-measured muscle activation in the flight muscles of insects.[98] We take the pulse song with constant amplitude in Figures 2.7a from a song simulation by Eberl[99] that has been used previously as a 'prototypical' *D. melanogaster* pulse song in playback studies.[99,100] Because constant song $S_c(t)$ can be any acoustic signal with constant amplitude, our model can also be used to study vocalization in other animals.

## 2.3 Discussion

Our modelling provides a framework for future experimental and theoretical work on courtship song. In Section 2.2 we state explicit assumptions of our model that are experimentally testable. In Section 2.2.1.2 we note that we assume the neuronal input to sine and pulse muscles to be similar. Moreover we pose that the neural input is integrated differently by the two muscles: the sine muscle has a steeper contraction slope than the pulse muscle. This assumption can be tested by visualising muscle activity of sine and pulse muscles (see Figure 2.2) with Calcium indicators while male flies sing.[20] We discuss how such a experiment could look like in Chapter 7 in Section 7.3. Furthermore we assume that the excitation at sine and pulse muscles is equally strong and equally patterned (see Figure 2.5). This assumption might be testable with Calcium Indicators, however the temporal dynamics of Calcium Indicators may be too slow to resolve APs.[20,101] Alternatively this assumption could be experimentally vetted with electromyographic recordings from sine and pulse muscles during song production.[48,51] Such experiments have been conducted and published in the 70s and it may be worthwhile to reproduce electromyographic recordings from song muscles with modern recording equipment.

In Figure 2.2 we map actual muscles and neurons to our model. More research is required to determine which muscles are active exclusively during sine or pulse song, and which muscles are active during both songs.[37,38] Similarly we require more work to understand the function of neurons in the patterning of song.[30,31] We show model muscle $x_{\text{psaf}}$ in Figure 2.2, which signifies muscles that are active both during sine and pulse song. We present this model muscle in our schematic as it is helpful for mapping actual muscles. However $x_{\text{psaf}}$ is not part of our mathematical formulation, nor of our MATLAB implementation of our model. Future work should consider implementing $x_{\text{psaf}}$.

We assume that muscle tension controls amplitude and predict characteristic variations in song amplitude, that we call song amplitude structures (SAS). We experimentally study SAS in Chapters 3, 4, 5, and 6. Our model thus motivates the experimental work in our thesis.

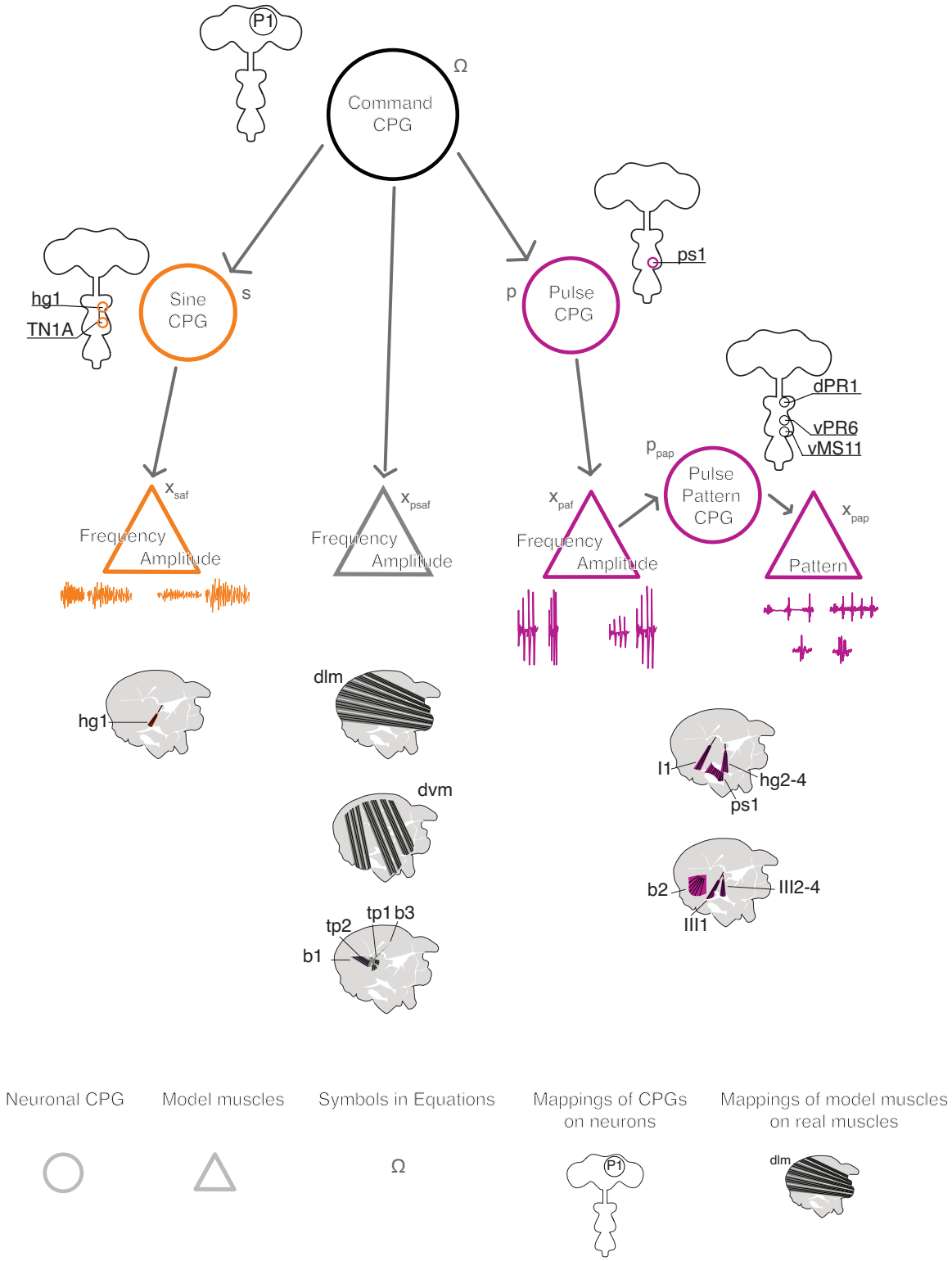



**Figure 2.2:** Schematic of song model.
The black circle stands for a command CPG which relays neuronal excitation to two low level CPG, a yellow circle for sine and a purple circle for pulse song CPG. The two low level CPG stimulate muscles (indicated by triangles) which subsequently contract. We map model CPG and model muscles to actual neurons and muscles. Note that muscle $x_{\text{psaf}}$ signifies muscles that are active both during sine and pulse song and is helpful for mapping real muscles to our model.

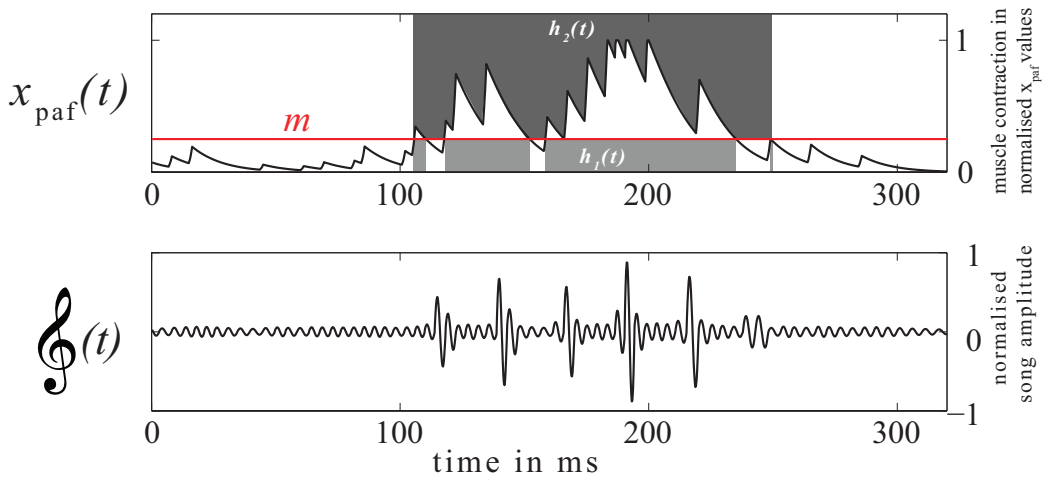



**Figure 2.3:** How is muscle contraction level $x_{\text{paf}}(t)$ related to pulse-amplitude pattern?
The helper function $h_1(t)$ indexes when $x_{\text{paf}}(t)$ exceeds muscle threshold $m$. The second helper function $h_2(t)$ smoothes over gaps smaller than $Z$ in $h_1(t)$. In the time window that is indexed by $h_2(t)$ the pulse-pattern neuron $p_{\text{pap}}$ fires. The firing of $p_{\text{pap}}$ yields pulse peaks and interspersed IPI as shown in the lower figure.

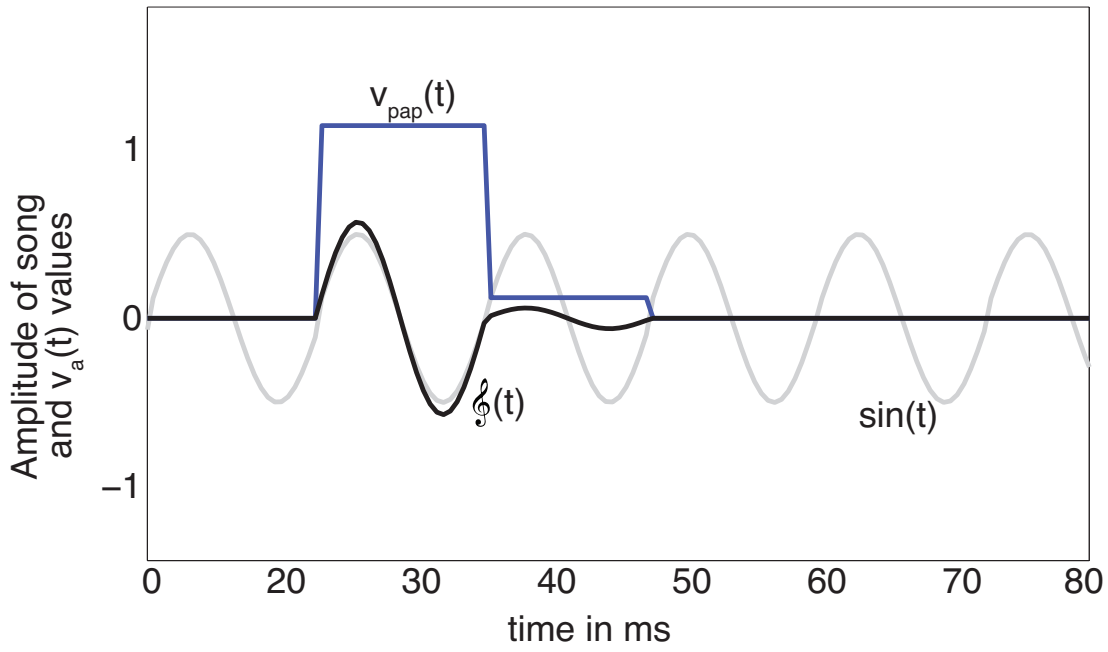



**Figure 2.4:** Illustration of sin function amplitude modulation to produce Drosophila courtship song patterns.
The mathematical sine function $sin(t)$ is multiplied with $v_{\text{pap}}$ to generate one pulse peak and subsequent IPI.

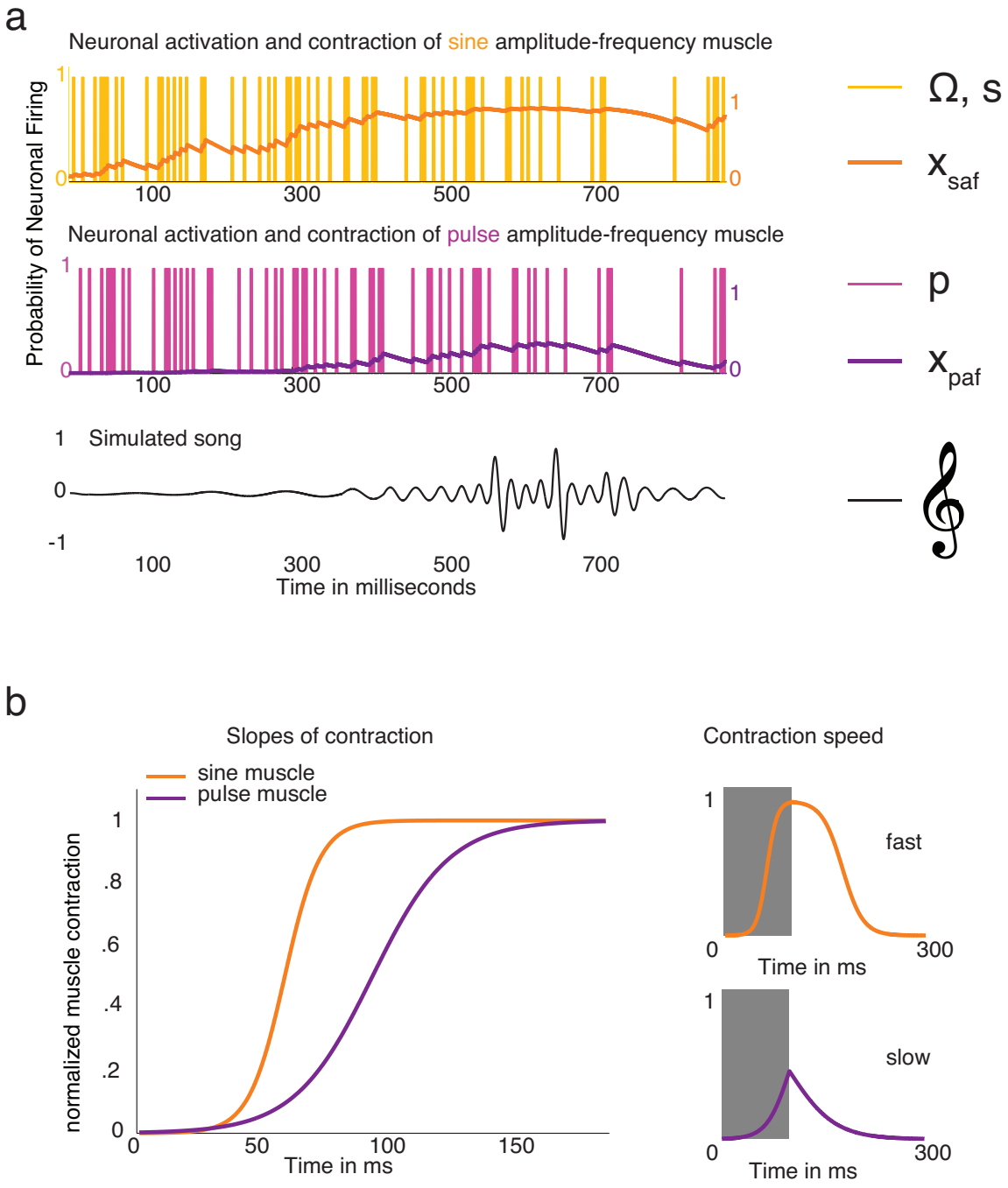



**Figure 2.5:** Illustration of characteristic courtship song model dynamics over time in response to neuronal excitation.

(**a**) Vertical lines represent neuronal activity in the sine (yellow) respectively the pulse CPG (pink). Thick lines represent muscle tension dynamics for amplitude and frequency muscles of sine (orange) and pulse (purple) that give rise to simulated courtship song as depicted in the lower graph. Sine and pulse muscles differ in their response to similar input, because they (**b**) differ in their contraction slopes such that contraction slope of the sine muscle $x_{\mathrm{saf}}$ is larger than $x_{\mathrm{paf}}$ of the pulse muscle. Due to this difference the same amount of neuronal excitation drives the sine muscle to full contraction and the pulse muscle to partial contraction.

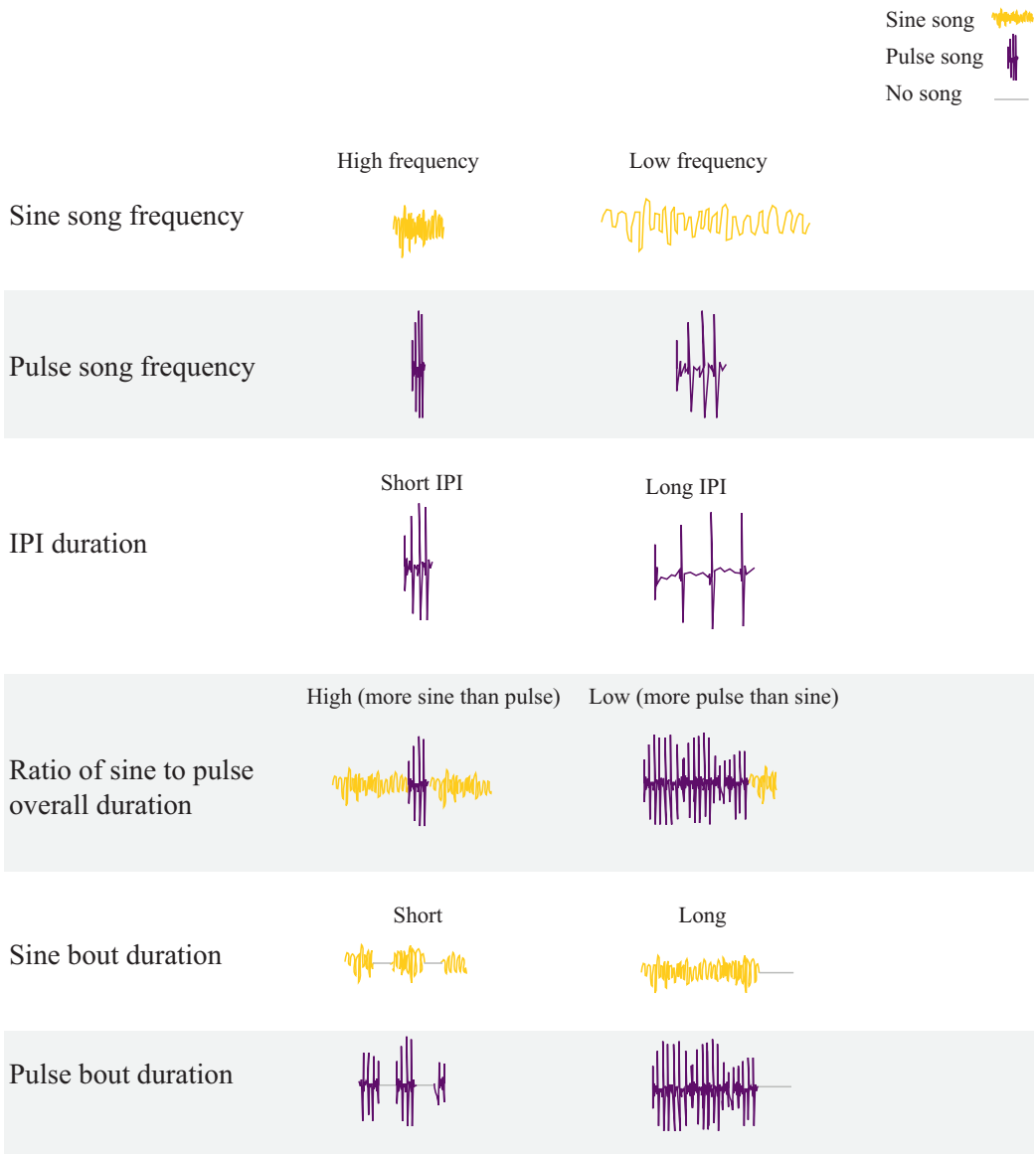



**Figure 2.6:** How can parameter settings affect courtship song?
Characteristic values for the six parameters in courtship song of *Drosophila melanogaster* can be found in Table A.1.

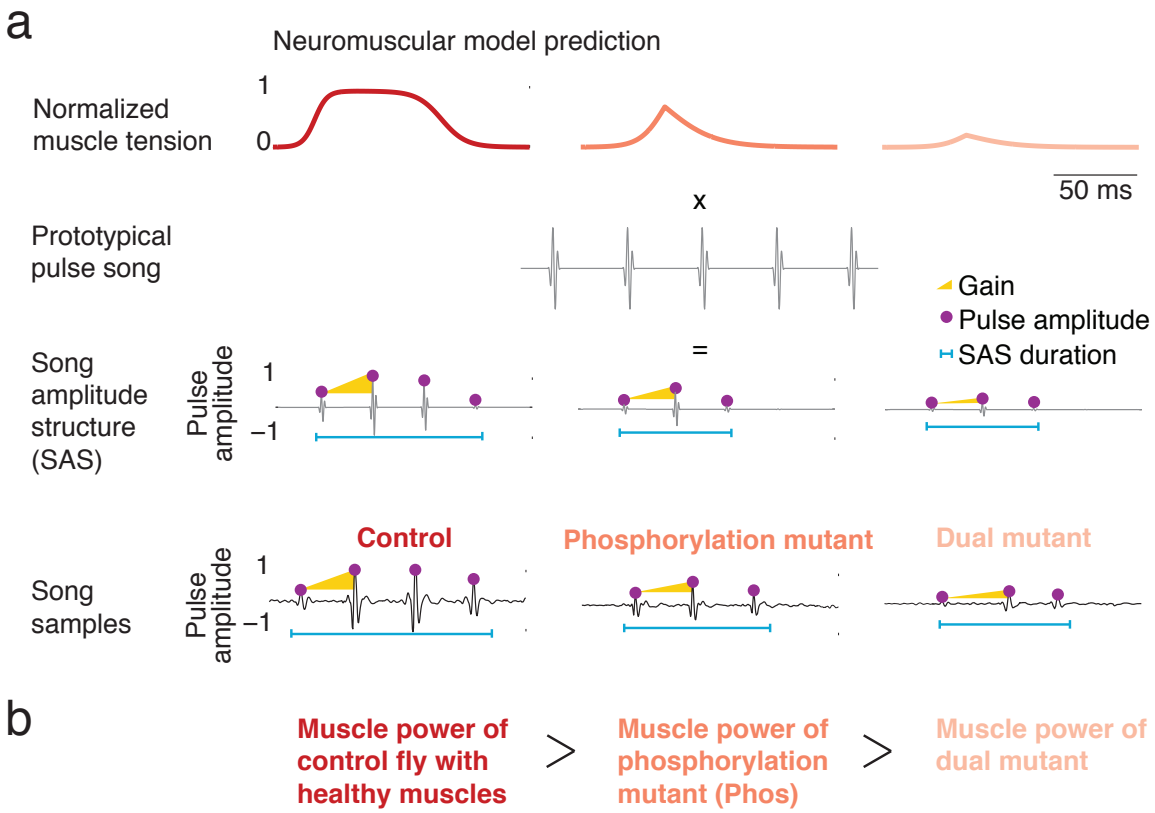



**Figure 2.7:** Distance-independent SAS may signal muscle neuromuscular dynamics.
(**a**) In our model, we assume that muscle dynamics are reflected by pulse SAS. We multiply simulated muscle tension functions with artificial constant-amplitude pulse song and thereby obtain song with variable SAS. Our model predicts that muscle power reductions are revealed by SAS, with flattened gain (yellow triangles), reduced amplitude (purple dots), and reduced SAS duration (blue lines). We give a precise definition of the SAS parameters in Section 3.2.3. (**b**) We tested the prediction of our model in *Dmlc2* mutants with reduced muscle power.

# Chapter 3

# Song amplitude structure in *Drosophila* is not explained by amplitude modulation with distance

## 3.1 Introduction

Amplitude modulations are common throughout the animal kingdom.[3,5,6] Amphibians,[6] insects,[5,31] birds[3] and mammals[9] modulate amplitude and these amplitude modulations can be processed by receivers[102] and some species consider amplitude in their mating decisions.[6,9] Recently Coen et al. found that male *Drosophila* modulate their song based on the distance to a female, singing louder when farther away from her.[31] This suggests that male flies adjust their amplitude to be heard by a female, which could explain the prominent amplitude modulations in *Drosophila* courtship song[31,43,45,51] (see Figure 1.1a). However AMD does not explain all amplitude modulations in courtship song[31] (Figure 3.1) and Coen et al. suggest that this may be due to choice of wing during singing: wing size affects song amplitude,[31,54] males switch wings during singing,[88] and males might have wings of different size. However Coen et al. note the technical difficulty of tracking which wing is used during courtship. It has not been tested so far whether wing choice affects song amplitude. Coen et al. also suggest that 'a physical limitation' may explain variance in song amplitude that cannot be explained by AMD.[31] 'A physical limitation' could refer to neuronal function, to muscle function, mobility of the wing hinge, ability of a wing to accelerate air particles and maybe other limitations and we do not know to which extent different physical limitations affect song amplitude relative to AMD.

Note that amplitude modulations are linked to muscle dynamics in the song of birds[14] and we suggest that muscle dynamics may be linked to amplitude modulations in *Drosophila* as well. Male starlings sing louder, the more their muscles are contracted. Similarly, when their muscles relax their song amplitude decreases.[14] One reason for the observed correlation of song amplitude and muscle activity in birds may be, that muscles control the power output of vocalisations.[14] In *Drosophila* the power output of the wing is correlated with activity of thoracic muscles,[20,101] which suggests that muscle activity may influence song amplitude in fruit flies. Indeed muscle activity of large indirect flight muscles correlates with song amplitude in flies.[31] This suggests that muscle activity and motor neuron activity may underly amplitude modulations in *Drosophila* song. Muscle activity depends on motor neuron activity in flies *Dickinson1999, Gordon2006, Lindsay2017* and motor neurons are connected with upstream CPGs and sensory neurons.[30,37,38] Variations in song amplitude may thus originate in variations in activity of upstream neurons, such as visual neurons.[31] In Chapter 2 Section 2.2.2 we present a neuromuscular model of song amplitude control, and based on it, we introduce the novel song parameter *song amplitude structure* (SAS). We investigate how much of amplitude variability can be explained by AMD and SAS and we test the prediction of our model that SAS is affected by muscle power.

## 3.2 Materials and Methods

### 3.2.1 Flies and experimental protocols

Unless we mention otherwise, we grew the flies at room temperature in a 12-hour light–dark cycle on standard cornmeal-based medium. We collected male and female virgin flies within six hours after eclosion. Before song recordings, males were aged in isolation for 3–7 days, and females aged in groups of eight animals of the same genotype for 3–7 days. For experiments, we placed one male and one female in a cylindrical mating chamber (of 10 mm diameter and 4 mm height). The floor of the mating chambers is a plastic grid that allows air vibrations to pass to the microphone. We paired couples of the same species. For recording song of muscle mutants, we paired a mutant male with a wild-type female (*D. melanogaster*, Oregon-R). The phosphorylation mutant ($Dmlc2^{S66A,S67A}$), the dual mutant ($Dmlc2^{\Delta 2\text{–}46;\ S66A,S67A}$), and the rescue line ($Dmlc2^{+}$) that we use as control are all transgenic lines in a null *mlc2*

background.[103] We recorded song between 0 and 11 hours after light onset. In each recording session, we recorded *Dmlc2*$^{+}$, *Dmlc2*$^{S66A,S67A}$, and *Dmlc2*$^{\Delta 2\text{–}46;\ S66A,S67A}$ flies from each genotype in various orders, which we determined at the beginning of each recording session. We did not predetermine sample size for song recordings.

### 3.2.2 Materials

We recorded song with a CMP-5247TF-K particle-velocity microphone[32] in a fly-song box[104] (see Figure 3.2). Our song box is equipped with a data-acquisition unit from National Instruments, which—together with a custom LabView script[104]— allows us to record fly song digitally. We recorded videos of flies during song recordings with a Stingray F-033C camera[105] and an NI PCIe-8253, IEEE 1394b Board[106] with Vision Acquisition. We designed mating chambers to come as one piece and to have a plastic grid as a base. (The plastic grid is more durable than a nylon mesh.) Our chambers can be printed on a 3D printer, which is a cheap and efficient alternative to manufacturing them manually. Our STL (Stereo Lithography) and solidwork files for 3D-printing of mating chambers are available at.[107]

### 3.2.3 Data analysis

#### 3.2.3.1 Song analysis

We automatically segmented recorded songs with FlySongSegmenter.[32] After song segmentation, we analyzed SAS gain, amplitude, and duration with MATLAB; our SAS analysis scripts are available at.[108] We now outline the workflow of SAS analysis:

1. Calculate amplitude maxima $\max(P_i)$ of individual pulses, where $P_i$ is the $i^{\text{th}}$ pulse as identified by FlySongSegmenter[32] in a song recording. The time $t_i$ is the time in milliseconds from the start of the song recording to $\max(P_i)$.

2. Determine local minima and maxima of pulse amplitudes $\max(P_i)$. We set the local-extremum identifier $L_i$ to 'local minimum' if a pulse is surrounded by two pulses with higher amplitude, so $\max(P_i) < \max(P_{i+1})$ and $\max(P_i) < \max(P_{i-1})$. We set $L_i$ to 'local maximum' if a pulse is surrounded by two pulses

with lower amplitude, so $\max(P_i) > \max(P_{i+1})$ and $\max(P_i) > \max(P_{i-1})$. For all other cases, $L_i$ is undefined.

3. Determine the initial and final indices of SAS. SAS is as an amplitude increase across a local amplitude peak followed by a decrease to a local minimum (see Section 3.3.1 text and Figure 2.7a), both the beginning and end of SAS are local minima. We set the SAS beginning and end identifier $S_i$ to 'start' if (1) $L_i$ is a local minimum or (2) $P_i$ is at the start of a pulse train. We set the SAS identifier $S_i$ to 'end' if (1) $L_i$ is a local minimum or (2) $P_i$ is at the end of a pulse train. The $i$ in $S_i$ signifies the count of identified SAS in a song recording. For all other cases, $S_i$ is undefined. We define pulse trains as in Coen et al. as consecutive trains of pulses with a temporal distance to adjacent pulse trains of at least 300 ms[401].

4. Calculate SAS slope as proportional amplitude change per time: the slope is

$$M_i = \frac{\max(P_i) - \max(P_{i-1})}{t_i - t_{i-1}} \qquad \text{for} \qquad i > 1\,.$$

   In our study, we focus on gain and thus only examine increasing slopes (i.e., slopes with $M_i > 0$).

5. Calculate amplitude as maximum of a pulse: $A_i = \max(P_i)$.

6. Calculate the SAS duration as the difference between SAS start and end indices:

$$D_i = t_i(S_i = \text{start}) - t_i(S_i = \text{end})\,.$$

#### 3.2.3.2 How much variance does AMD and SAS explain?

We compute $R^2$, which gives the fraction of variance explained by SAS:

1. Detect SAS in recordings of fly courtship song as described above.

2. Fit models to each detected $\text{SAS}_i$ to calculate $R_i^2$ for $\text{SAS}_i$.

[1]The choice of a temporal distance of 300 ms is arbitrary and could be made differently. When one makes a choice for what temporal distance to expect between trains of pulses one should consider at least two questions: (1) Does my choice result in more pulse trains than one would detect manually? (2) Does my choice result in less pulse trains than one would detect manually?.

3. Take a mean over all $R_i^2$ to obtain $R^2 = \frac{1}{n_{\mathrm{SAS}}} \sum_i^{n_{\mathrm{SAS}}} R_i^2$, where $n_{\mathrm{SAS}}$ is the number of detected SASes in our analyzed courtship songs.

We fit three types of regression models to SAS: sigmoidal, exponential, and linear. Sigmoidal and exponential regression models are reasonable choices, because the neuromuscular dynamics can produce sigmoidal and exponential patterns (see Figure 2.7). We also fit a linear regression model to SAS as a baseline control for the nonlinear regression models. We find that the linear ($R^2 \approx 0.86$, $n = 801$, where $n$ is the number of SAS fitted by this model) and the exponential regression models ($R^2 \approx 0.91$, $n = 801$) produce a better fit than the sigmoidal regression model ($R^2 \approx 0.68$ $n = 801$), which suggests that SAS is not well-represented by sigmoids. However the relatively bad fit of sigmoidal regression models compared to the linear and exponential regression models may be due to the preponderance of short SAS. In fact more than 65% of detected SAS consist of three or fewer pulses and for fitting a regression model to data, the number $x$ of data points must be at least the number of parameters. Because the sigmoidal model has DF = 4 parameters (minimum, maximum, offset, and slope), we analyze only SAS in which the number of pulses is $x \geq 4$. This excludes about 65% of SAS from the regression analysis because of statistical restraints. Why do we find many SAS with less than four pulses? The reason for this may be our characterisation of SAS: SAS start and end points are local minima in song amplitude, and small dips in song amplitude may be due to noise. However a small dip in amplitude can result in a local minimum, which then signifies the start of a new SAS. Therefore, our measurement of SAS may result in SAS of short durations, which agrees with our finding that a significant portion of detected SAS (about 37%) in *D. melanogaster* songs consists of two or fewer pulses.

We find that exponential regression models fit SAS somewhat better ($R^2 \approx 0.91$, $n = 801$) than linear baseline control models ($R^2 \approx 0.86$, $n = 801$), which suggests that SAS has a nonlinear component (as one would expect to arise from neuromuscular dynamics) that is captured by exponential dynamics. However, the fit of linear regression models is almost as good as that of the exponential model, and linear song amplitude envelopes seem to do a reasonable job of capturing natural SAS.

The $R^2$ values that we report are only rough estimates of the fraction of variance explained by SAS, because we use regression models instead of our original neuromuscular model[109] to estimate the fits. We do this to try to minimize the number of parameters that need to be estimated. Our original neuromuscular model has a total of 12 parameters,[109] which we find to be intractable for parameter estimation with ABC–SMC (Approximate Bayesian Computation with Sequential Monte Carlo

Sampling),[110,111] as the parameter estimation did not converge after 6 months on 32 parallel processors.

## 3.3 Results

### 3.3.1 Can amplitude modulation with distance explain amplitude variation at constant distances?

We reanalyzed the data of Coen et al.[31] and also found that AMD cannot account for all of the variability in male song amplitude. (See Section 1.5 for a review of Coen et al.[31] and see Figure 3.1b for our reanalysis of their data.) We find a mean Pearson correlation coefficient of song amplitude and distance between flies of $R^2 \approx 0.05$ across flies (see Section 3.2.3 for details) which suggests that AMD can explain about 5% of the amplitude variation. While AMD explains a small portion of variance in amplitude, we note that distance correlates significantly with song amplitude, as reported previously[31] in about about 82% of flies. (The p-value is $P < 0.001$ from linear regression on song and distance data of 315 flies using the data set of Coen et al.[31]) However AMD does not explain amplitude variations at constant distances. These variations could be due to noise in biological systems.[31] However, we find that distance-independent variations in amplitude are not uniformly random ($P < 0.0001$ and a $\chi^2$-goodness-of-fit test; see Figure 3.3), which suggests that uniformly random noise cannot explain amplitude variations at constant distances.

### 3.3.2 Song amplitude structure may explain amplitude variation at constant distances

Changes in amplitude that cannot be explained by dynamic distance estimations can perhaps be explained by neuromuscular dynamics, which are inherent to song-generating systems.[31,48,51] The simplest type of relationship between muscle force and song amplitude is a linear one (see Figure 2.7a), and we assume this for illustrative purposes.

As expected from our simple neuromuscular model, we observe variations in song amplitude when distance is constant. (We specify our model in Section 2.2.2 and illustrate our model in Figure 2.7a.) We use the term "song amplitude structure" (SAS) for these diamond-like pulse amplitude patterns, which cannot be explained by distance between flies and which complement the notion of AMD.[31] We observe

SAS as an amplitude increase across a local amplitude peak followed by a decrease to a local amplitude minimum, which in our model corresponds to muscle contraction followed by muscle relaxation.

We find that about 91% of amplitude variance in pulse song can be explained by SAS (see Section 3.2.3). Additionally, mechanical restraints in a thorax and a wing hinge may affect how neuromuscular dynamics are translated into wing vibration,[95] possibly constituting another source of variance that we do not incorporate in our model.

### 3.3.3 Song amplitude structure signals muscle power in *Drosophila*

Our model predicts that reduced muscle power affects SAS, and we wish to test this prediction (see Figure 2.7b). If we assume that the muscles that generate different powers are activated at similar levels,[74] our model predicts that the gain, amplitude, and duration of SAS decrease with decreasing muscle power (see Figure 2.7a). Gain is the increase in amplitude per second, amplitude is the maximum peak of a pulse, and duration measures the time it takes for amplitude to increase across a local amplitude peak and then decrease to a local amplitude minimum. (For illustrations of measurement of SAS duration, amplitude and gain, see Figure 3.4a–c.) To test our prediction, we analyze song from males with mutations known to decrease muscle power.[74] Mutations in the *Drosophila myosin regulatory light chain* (*Dmlc2*) are specific to muscles and are known to reduce muscle power by changing the molecular structure of the myosin head and its position relative to the actin binding site without severely affecting the ultrastructure of the muscle fibers.[74] We record male song from a rescued $Dmlc2^{+}$-null control, the phosphorylation mutant $Dmlc2^{S66A,S67A}$, and the dual mutant $Dmlc2^{\Delta 2\text{–}46;\ S66A,S67A}$ (see Figure 2.7a). As predicted, $Dmlc2^{S66A,S67A}$ and $Dmlc2^{\Delta 2\text{–}46;\ S66A,S67A}$ exhibit significantly lower SAS gain, duration, and amplitude than the controls (see Figure 3.4d, Figure 3.4e, and Figure 3.4f). All other pulse song parameters that we examine—IPI, cycles per pulse (CPP), and pulse frequency—do not differ consistently between mutants and control[43] (see Figure 3.5), but SAS appears to help reveal muscle power. Notably, our model assumes that muscle tension development is revealed specifically in variations of song amplitude. The ratio of mean sine song amplitude to mean pulse song amplitude is larger in *Dmlc2* mutants than in controls,[43] suggesting that muscle power reproducibly affects song amplitude. Moreover, *Drosophila* flight muscles control pulse amplitude,[31] supporting our observation that muscle power relates directly to SAS.

## 3.4 Discussion

Our analysis suggests that song amplitude correlates positively with muscle dynamics. In birds, it has been demonstrated that song amplitude correlates positively with electromyographic (EMG) recordings of vocalizing muscles,[14] but this has yet to be demonstrated for thoracic muscles in *Drosophila*.[31,48] Recent advancements in imaging techniques allow one to measure muscle activity in live fruit flies.[20] It would be interesting to record *in vivo* muscle activity of a singing male and at the same time record his song amplitude and thus gain insight in the relationship between song amplitude variations and muscle dynamics in *Drosophila*.

We presented a statistical comparison between AMD and SAS, which is flawed as the underlying models of AMD and SAS differ in their number of parameters: AMD has one parameter (distance) and in our analysis of Coen's data SAS has 801 parameter. (See Section 3.2.3 for details of our analysis of Coen's[31] data.) Thus our finding that SAS fits amplitude data may be due to overfitting data. However, our finding that AMD explains only about 5% of amplitude variation illustrates that the majority of amplitude variation is not explained by AMD and we suggest that SAS may be a useful concept to study amplitude variations.

One could record muscle activity[20,101] and song amplitude[31] from a male *in vivo* while he is freely moving and courting a female.[18] When combined with fly tracking,[18] such a setup would allow an experimental comparison between AMD and SAS, because both distance and muscle activity between flies can be correlated with song amplitude.[31] This may allow an estimation of how much variation in amplitude is explained by distance between flies and how much variation is explained by muscle activity. Vision is likely a sensory source of muscle activity control, and we believe AMD and SAS are complementary. However, they also need to be distinguished to gain a better understanding of song amplitude control and perception.

Importantly, our neuromuscular model and the AMD model[31] are not in conflict with each other: we suggest that variations in song amplitude can be explained my neuromuscular variations and Coen et al. suggest that variations in song amplitude can be explained by visual estimations of distance. They show that such distance estimations affect neural and muscular activity and song amplitude.[31] This is in agreement with our model, in which we assume that neuromuscular activity affects song amplitude. Importantly AMD explains only about 5% of amplitude variations, leaving a majority of variations unexplained. One reason may be that the circuit for song amplitude control is not well understood and requires further investigation.[31]

AMD fails to explain amplitude variations at constant distances, which are observed in real song (see Figure 3.1). When distances are stable, visual stimulation is constant and one may expect constant song amplitude,[31] but this is not the case in song recordings. Thus we require an explanation for amplitude variations at steady stimulation. Our model offers such an explanation: amplitude variations at constant stimulation may be a by-product of neuromuscular transforms.[97] When a male fly sees a female fly at a distance, his song-control system sends a neuronal command to the relevant muscles to initiate louder singing.[31] Following stimulation, the muscle generates tension incrementally.[74] When the muscle contracts thus, male flies may sing louder. Importantly this assumes that muscle activity and song amplitude are correlated. Currently, this relationship between muscle force and song amplitude is not experimentally testable in *Drosophila*, as it would require one to measure muscle-force dynamics *in vivo* in singing flies. Thus far, muscle-force dynamics in insects have been measured *in vitro* and *in situ*[74,98,112] but not *in vivo*. However Calcium indicators can be measured *in vivo* in flies and they are an approximation of muscle-force dynamics.[20,101]

We predicted specific changes in song amplitude in flies with reduced muscle powers. Indeed we find the predicted changes in muscle mutants of the *Dlmc2* gene, that have reduced muscle power.[74] Notably, these muscle mutants have been independently shown to sing with reduced amplitude,[31] which implies that this finding is robust across laboratories. Our findings add to the previous work, by including measurement of SAS gain and SAS duration, which are affected in these mutants. We will demonstrate in Chapter 4 that SAS gain can affect female mating decisions and is thus important.

We find that other song characteristics like IPI, CPP and frequency do not consistently differ between muscle mutants and controls (see Figure 3.5). We show that the Phosphorylation mutant differs in frequency and IPI from controls and Dual mutants, which might suggest that IPI and frequency are affected by muscle power. However if true, the Dual mutant should show similar deviations as the Phosphorylation mutant, which is not the case however. Thus we conclude that the observed differences in IPI and song frequency may be not due to differences in muscle power. Note that IPI, CPP and frequency have been measured in an independent lab in these mutants.[43] They found that these song characteristics do not differ consistently between *Dlmc2* mutants and controls,[43] which is in agreement with our results. One explanation for the robust finding that muscle power affects song amplitude,[31] but does not affect other song features,[43] may be that muscular control of song amplitude and other song

features is functionally independent (see Figure 2.5 for a mapping of muscles that may control song amplitude and other song features as IPI and CPP). Note that muscle power of large indirect muscles has been associated with song amplitude control.[31] These muscles also have been shown to have reduced muscle power in *Dlmc2* mutants.[74] However there are no published data on how *Dlmc2* mutations affect small direct muscles, that might be controlling song features as IPI, CPP and frequency.[37]

Song amplitude can be actively controlled by male flies,[31] and our work suggests that SAS signals biologically meaningful information (muscle power). Female flies may respond to variations in song amplitude, and this should be investigated in future research.

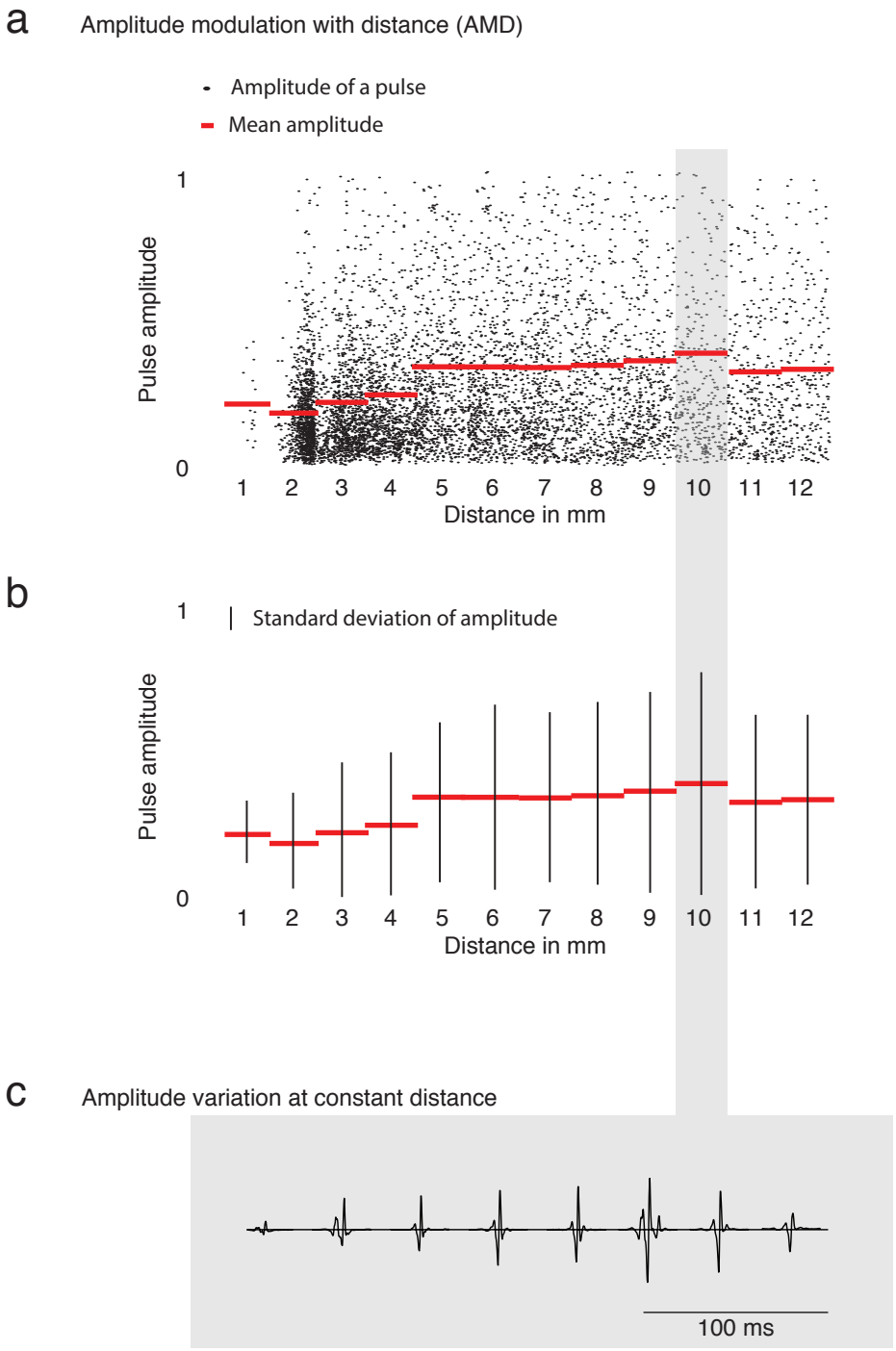



**Figure 3.1:** Distance-independent song amplitude variations.
**(a)** Amplitude modulation with distance (AMD) predicts constant song amplitude at constant distances between flies,[31] although song amplitude is variable at constant distances. We show dot plots of amplitudes at distance intervals of 1 mm (except for 1.5 mm for the initial interval), which is approximately half of a body length in fruit flies. (See Section 3.2 for details on distance categories.) Each dot represents the amplitude of one pulse. Red bars mark the mean amplitude for a given distance interval. **(b)** Similar as **(b)**: we show mean and standard deviation of pulse amplitudes for each bin plotted against distance. **(c)** The displayed song at distance category '11' is a recording from Coen et al.[31] We indicate the amplitude of the song pulses with purple dots. We also show the purple dots in the dot plot in (**a**) to illustrate amplitude variation at a relatively constant distance.

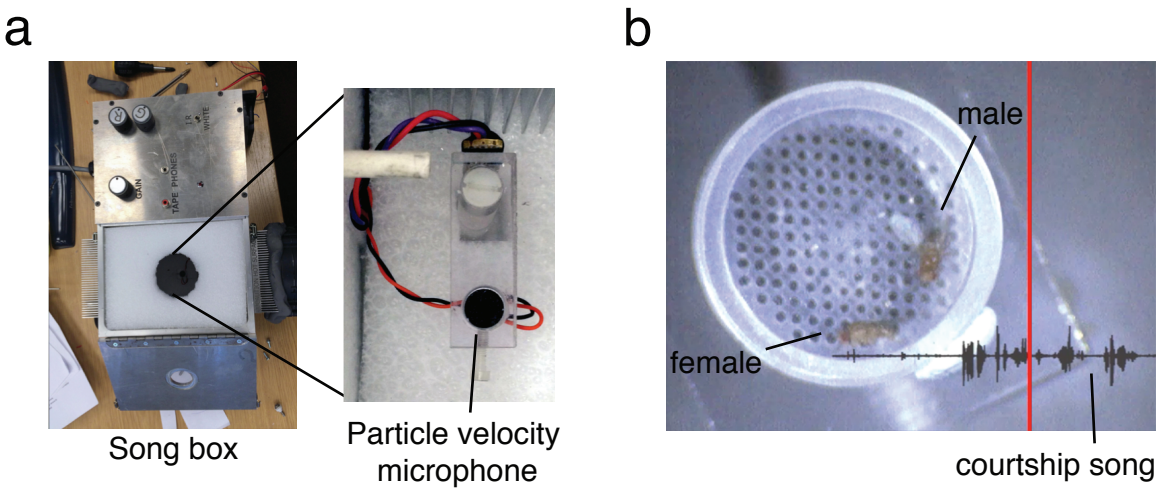



**Figure 3.2:** Our setup for recording courtship song of *Drosophila*.
(**a**) In our recording setup we use a particle velocity microphone to record courtship song. Our song box[104] is fitted with styrofoam to reduce noise interference from outside. (**b**) We place a courtship chamber with perforated bottom on our microphone to record song from a male fly who is courting a female fly.

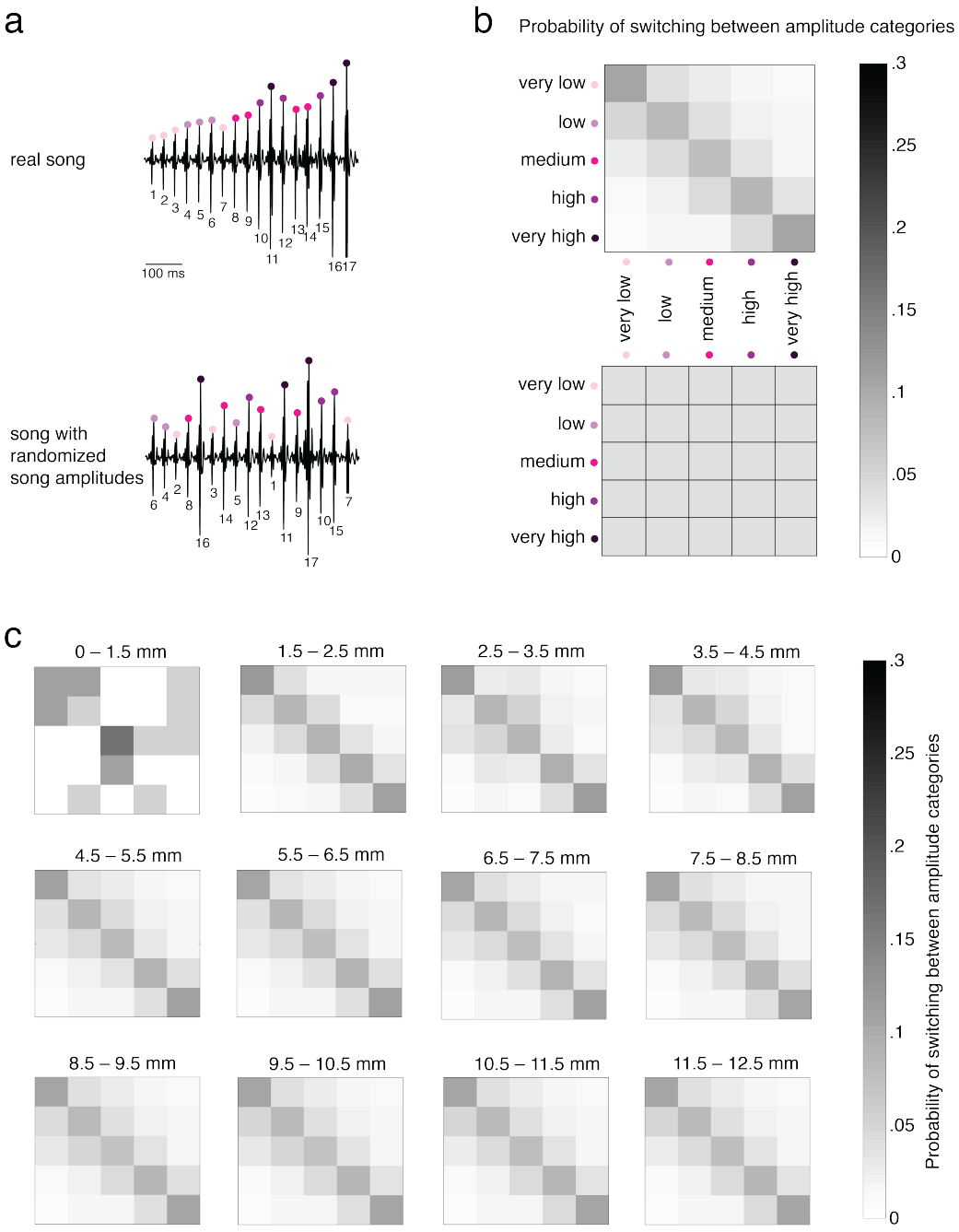



**Figure 3.3:** Distance-independent variations in song amplitude are not uniformly random **(a)** Recorded courtship song of a *Drosophila melanogaster* male. Dots indicate the maximum amplitude of pulses. The colors of the circles represent amplitude categories from very low amplitude (light pink) to very high amplitude (purple). The same recording with a randomized sequence of song amplitudes. (The randomization uses Mersenne Twister with the seed set to 0.) The numbers below each pulse illustrate the randomization. **(b)** Heat maps reveal the empirical probabilities of switching between amplitude categories in Coen et al.'s song data.[31] Each amplitude category includes a similar number of peaks. Therefore, if amplitude variations were uniformly random, the probability of switching would be approximately uniform across amplitude categories. The switching probabilities in real song (at distances of 11.5–12.5 mm) differ significantly from random (the p-value is $P < 0.0001$ using a chi-squared goodness-of-fit test). **(c)** The switching probabilities in real song for all tested distance intervals differ significantly from random ($P < 0.0001$, chi-squared goodness-of-fit test). The amplitude category labels are the same as in panel (b).

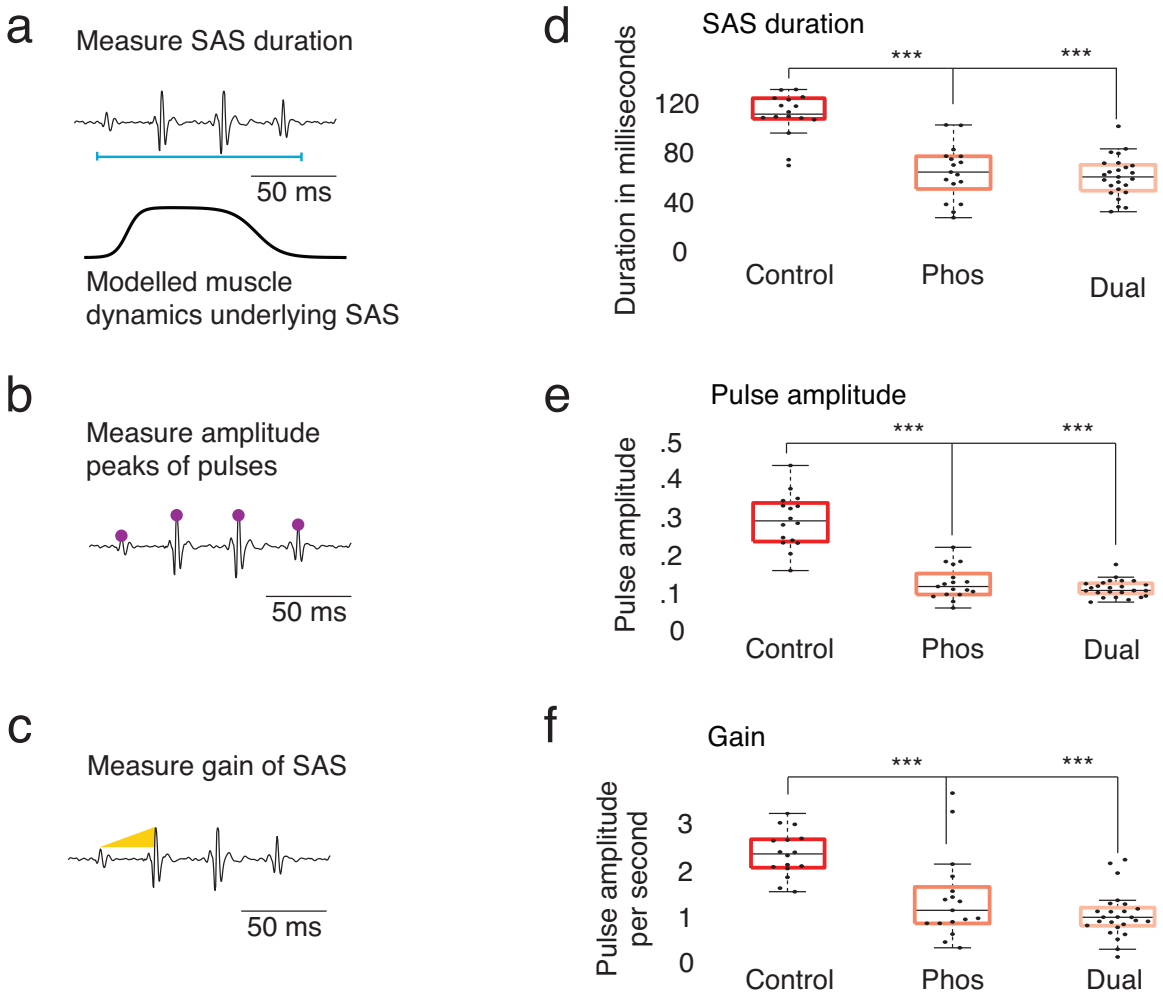



**Figure 3.4:** Distance-independent song amplitude variations signal muscle power and can be explained by neuromuscular dynamics.

(**a**) We illustrate the measurement of SAS duration (blue line) and indicate the underlying model of muscle dynamics. (**b**) We also illustrate the measurement of amplitude peaks (purple dots) and (**c**) SAS gain (yellow triangles). (**d**) box plots of SAS duration, (**e**) pulse amplitude and (**f**) SAS gain for controls and *Dmlc2* mutants (which have reduced muscle power). The horizontal line indicates the median. Bee plots illustrate distributions of the SAS parameters; each black dot represents an individual fly's mean. SAS duration, peak amplitude, and SAS gain are significantly smaller in phosphorylation mutants ($n_s = 15$, where $n_s$ is the number of songs from individual flies) and dual mutants ($n_s = 26$) than in controls ($n_s = 16$) with healthy muscles. The asterisks indicate significance levels: *** indicates $P < 0.0003$ (after Bonferroni correction for multiple testing using a two-sided Wilcoxon signed-rank test, WR). See Section 3.2.1 for more information on the muscle mutants.

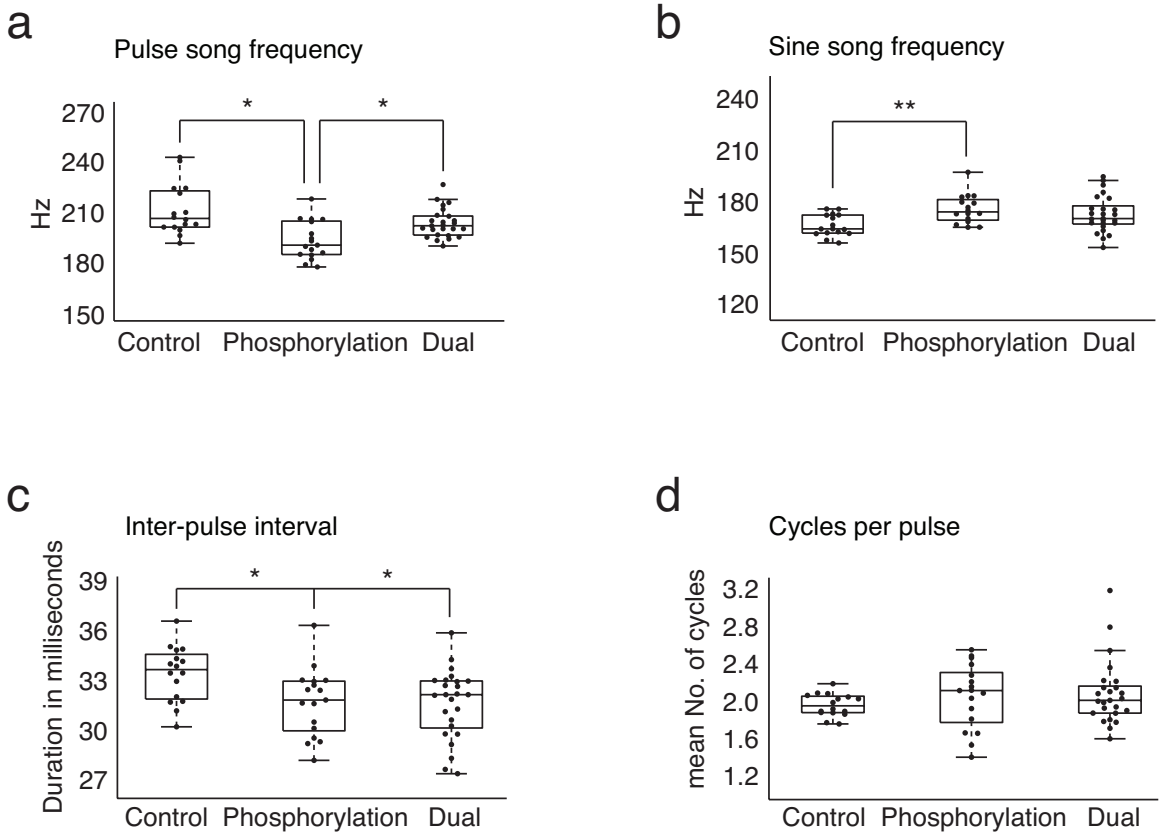



**Figure 3.5:** Comparison of song frequency, inter-pulse interval and cycles per pulse in controls and muscle power mutants.[74]

**(a)** Box plots of pulse song frequency in control flies (rescued *Dmlc2*$^{+}$-null control), *Dmlc2* muscle mutants (phosphorylation mutant *Dmlc2*$^{S66A,S67A}$, and the dual mutant *Dmlc2*$^{\Delta 2\text{–}46;\ S66A,S67A}$). In the box plots, the horizontal line indicates the median, the box indicates the inter-quartile-range (IQR), and the whiskers indicates 1.5 × IQR. We overlay box plots with bee plots to illustrate the distributions. Each black dot represents an individual fly's mean song parameter. **(b)** Box plots of sine song frequency in controls and in *Dmlc2* muscle mutants. **(c)** Box plots of IPI in controls and in *Dmlc2* muscle mutants. **(d)** Box plots of cycles per pulse in controls and in *Dmlc2* muscle mutants. The asterisks indicate significance levels, with * indicating $P < 0.016$, ** indicating $P < 0.003$, and *** indicating $P < 0.0003$ after Bonferroni correction for multiple testing using WR.

# Chapter 4

# *Drosophila* song amplitude structure is a communication signal

## 4.1 Introduction

As we discussed in Chapter 1, it was shown recently by Coen et al.[31] that male flies actively modulate their song amplitude relative to distance from females. Specifically, males sing louder when a female is farther away, suggesting visual control of acoustic communication in *Drosophila*. However, distance explains only a small portion of amplitude variability (see Chapter 3), and residual amplitude variance may be due to muscle dynamics (see Chapters 2 and 3). One can quantify patterns in amplitude variations to show that SAS signals muscle power (see Chapter 3). These results suggest that song amplitude can be actively controlled by male flies and that SAS signals biologically meaningful information. We now examine whether SAS influences courtship behaviour in flies.

Females from the sibling species *D. melanogaster* and *D. simulans* use characteristics of male songs, such as IPI, to choose mating partners.[70,94] Females prefer to mate with males from their own species and can differentiate between different IPI.[70,94] We examine whether females also use SAS[62] for making mating decisions, and we find *D. melanogaster* and *D. simulans* females do this.

As is standard, we assess auditory responses of male flies with male chaining in response to song playback.[66,72,99,100] Chaining is a behavioural phenomenon in which male flies start to court each other, forming courtship chains.[99] Wildtype males typically chain upon hearing a song with their species characteristics.[66,72]

It is not obvious why male flies court other males. In fact, male-male chaining is arguably a waste of resources, as males spend energy on courtship without the possibility to reproduce. Thus chaining seems to have no clear evolutionary benefit. However homosexual displays have been presented as indirectly evolutionary beneficial, in being a by-product of decision-making on whom to court.[113,114] Male and female flies look similar and may be confused. Identification of the evolutionary correct target for courtship (the female) may not be self-evident for males, and they may make decisions in uncertainty. Being uncertain whether to court, *Drosophila* males could chose at least two strategies: (1) do not court and (2) court. If their strategy was to not court when uncertain, they could miss females, and thus the opportunity to pass on their genes, which is evolutionary detrimental. However if they chose to court when uncertain, they would run less danger to be un(re)productive, with the cost of higher energy expenditure. Courting other males may be less disadvantageous for species survival than not courting females. While decision-making in uncertainty may explain why male-male courtship is evolutionary stable,[113,114] it does not fully explain chaining in flies. Chaining behavior is triggered by the sound of male courtship song. Notably only males sing in most *Drosophila* species,[85] which suggests that a male-specific behavior triggers courtship in other males. As only males sing, it is unlikely that males mistake song as a female signal. This raises the question, why song stimulates males to chain. One reason could be that song acts as a cue for females. If a male sings, a female may be close-by, and other males might take notice of the females presence by hearing courtship song. However such a cue may be treacherous, as males sometimes court other males. Thus song may act as uncertain signal for females.

As with females flies, males can differentiate between different IPIs, and they respond most to song with their own species IPI.[66,72] Male *Drosophila* perform elaborate courtship dances for other flies, including other males. Researchers call this homosexual display of fly males *chaining* and study it to better understand courtship and perception.[66,99,100] We examine whether male flies can also differentiate between different SAS, and we find that male *D. melanogaster* and *D. simulans* indeed differentiate SAS, suggesting that SAS is a communication signal for both male and female flies.

## 4.2 Materials and methods

### 4.2.1 Flies and experimental protocols

Unless we mention otherwise, we grew the flies at room temperature in a 12-hour light–dark cycle on a standard cornmeal-based medium. We collected male and female virgin flies within six hours after eclosion. In playback experiments, we controlled sound levels throughout playback with a CEL-246 sound-level meter. Because we used playback stimuli with variations in amplitude, sound levels varied between about 85 dB and about 100 dB. We controlled for mean playback amplitude by generating playback stimuli that have similar mean amplitude (less than ±1% amplitude difference between playbacks).

Before song recording and playback experiments, males were aged in isolation for 3–7 days, and females aged in groups of eight animals of the same genotype for 3–7 days. For song recording, we placed one male and one female in a cylindrical mating chamber (of 10 mm diameter and 4 mm height). The floor of the mating chambers is a plastic grid that allows air vibrations to pass to the microphone. We paired couples of the same species, and we recorded song throughout the day. In each recording session, we recorded *D. melanogaster*, and *D. simulans* flies from each species in various orders that we determined at the beginning of each recording session. We did not predetermine sample size for song recordings.

For copulation assays, we muted and deafened male flies by cutting their wings and arista.[29] Male flies in the copulation assay were thus unable to hear song playback, whereas females were able to hear it. The copulation assay thus pertains to female responses to songs, as deafened males do not respond to song.[100] We conducted copulation experiments throughout the day, and we did not use blinding for analyzing playback assays.

We followed the chaining protocol described in.[100] We grew and aged *D. simulans* males on a rich cornmeal-based medium, as we found that this reduced their baseline chaining levels. We collected male virgins within six hours after eclosion and muted them by cutting their wings above the anterior crossvein. We conducted chaining experiments between 0 and 4 hours after light onset and determined the alternating order of playbacks at the beginning of each session. We predetermined sample sizes for chaining experiments with an online sample-size calculator.[115] The sample-size calculator requires one to specify effect size (Cohen's $d$) and $\phi$, which is the probability that the null hypothesis is correctly rejected, to calculate sample

size. We used the default value $\phi = 0.8$ for sample-size calculation and determined the effect size of chaining in our setup in a pilot study. In the pilot study, we played back real songs with species' mean gain envelopes (see Figure 4.3b for illustration of playbacks with species mean gain envelopes) to the two species and found effect sizes between approximately 1.0 for *D. simulans* (with a sample size per group of 17) and approximately 1.4 for *D. melanogaster* (with a sample size per group of 10).

### 4.2.2 Materials

We recorded song with the equipment described in Chapter 3 . In line with prior experiments,[43,54–56,116] we record song with a single microphone and a male singing on top of this microphone. We control for distance to the microphone by using a setup in which a male is always on top of the microphone, instead of one in which a male can distance itself from the top of the microphone.[31,40,117] Microphone sensitivity can vary across a microphone, so the position of a male on the microphone may affect recorded song amplitude. We do not have data on the position of males and thus cannot control for such position effects, so this is a limitation of our study.

We played back song with a Mach sub bass speaker, which is connected to an EP-800 amplifier, which in turn is connected to a creative sound blaster X-Fi Xtreme audio PCI sound card in an OptiPlex 3020 mini tower PC. We used Windows Media Player (version 12.0.7601.19148) with the default setting for all playback. We placed flies for playback experiments in chambers, with rectangular cross sections, that are similar to the chambers described by,[100] although ours have a plastic grid for front and back walls instead of nylon mesh. After placing flies in our playback chambers, we positioned them on a box of 30-centimeter height to level the flies at the speaker center. We illuminated the flies from below with a comic master light table A4.[118]

### 4.2.3 Data analysis

We automatically segment recorded songs with FlySongSegmenter.[32] This song analysis methodology[32] has been criticised recently,[33] but we find that FlySongSegmenter detects about 95 % of pulses that we manually detect. Additionally, FlySongSegmenter has been widely accepted in the fly song community as software for song analysis.[31,40,117,119] After song segmentation, we examine SAS gain, amplitude, and

duration with MATLAB. SAS gain is the increase in amplitude per second, amplitude is the maximum peak of a pulse, and duration measures the time it takes for amplitude to increase across a local amplitude peak and then decrease to a local amplitude minimum. (See Section 3.2 for a mathematical definition of SAS gain, amplitude and duration.)

We manually count the number of copulating couples during 20 minutes of playback. Our two-sample comparisons are two-sided Wilcoxon rank-sum tests (WR), as the sample distributions are non-normal. When appropriate, we correct for multiple testings using Bonferroni correction.

For chaining experiments we assess baseline chaining levels during 5 minutes of silence which are then followed by 5 minutes playback, as described previously.[66,99,100] We examine chaining with the tracker software ChaIN.[66] We pool chaining over the 5 minutes of playback and then compare chaining levels between playback conditions. ChaIN counts the number of males in a chain at a given second; this quantity is called the 'chaining index'.[66] There are 6 males per group, so the maximum number of chaining males is 6 (and the minimum number is 0). Our analysis is based on raw chaining data that includes 0 values, which reduces the overall chaining index. We manually exclude data sets with unusually high levels of chaining during silence. Based on our experience, we judge normal baseline chaining to be no more than 2 on average, which is also what other researchers have reported for baseline chaining.[66,100]

### 4.2.4 Stimulus design

For the experiments that we present in Figures 4.5, 4.6a, 4.7b, and 4.7c, we design playback stimuli from recordings of vigorous courtship song of *D. melanogaster* and *D. simulans*. (See Figure 4.3c for stimuli design; for playback statistics, see Figure 4.8.) For the experiment that we present in Figure 4.6b, we modulate SAS in artificial *D. melanogaster* song generated by Joerg Albert (Ear Institute, University College London). We control the mean amplitude of playback stimuli by computing the mean amplitude of each playback stimulus and subsequently adjusting the mean amplitudes to be less than $\pm 1\%$ different from each other, by identifying the playback $p_A$ with the highest mean amplitude $A$ of playbacks in an experimental condition and then calculating the relative difference in mean amplitude $\Delta A = \frac{A}{a_i}$, where $a_i$ is the mean amplitude of playback $p_i$ in an experimental condition. We then multiply playback $p_i$ by $\Delta A$, such that $p_\Delta = p_i \times \Delta A$. We share our MATLAB code[120] for generating playback stimuli from *Drosophila* audio tracks and now outline the workflow:

1. Detect pulses $P_i$ with FlySongSegmenter.[32]

2. Normalize pulse amplitudes to generate a piecewise-constant amplitude audio track $S_c(t)$. Pulse-amplitude maxima and minima can have different absolute values, but we set them to the same absolute value after normalization. We normalize maxima and minima separately in two steps. For normalizing maxima, we (1) determine the pulse maxima $A_\mathrm{i} = \max(P_i)$. We then (2) split the audio track $S(t)$ along the horizontal axis (i.e., $t$-axis) to generate split audio tracks, which represent positive or negative amplitude portions, to separately normalize amplitude maxima and minima. We generate a split audio track $S_p(t)$, which consists of 0 and all positive values of $S(t)$. (3) We normalize pulses in the split audio track $S_p$ by dividing them by their amplitude maxima $A_i$. For (4) normalizing pulse amplitude minima, we determine the pulse minima $\min(P_i)$. We then (5) split the audio track $S(t)$ along the $t$-axis to generate $S_n$, which consists of all negative $S(t)$, and we (6) normalize pulses in the split audio track $S_n$ by dividing them by the absolute value of their amplitude minima $|\min(P_i)|$. Finally, we (7) join the normalized split audio tracks $S_p$ and $S_n$ to generate a constant-amplitude audio track $S_c(t)$, such that $S_c(t) = S_p$ for all $t$ that satisfy $S(t) \leq 0$ and $S_c(t) = S_n$ for all $t$ with $S(t) < 0$.

3. Make amplitude masks as functions of SAS gain. Because masks with 0 values can set pulse amplitudes to 0 and hence effectively erase pulses, we allow masks to take values between 0.2 and 1 (i.e., the mask $m_s \in [0.2, 1]$), as we find that flies respond to pulses when we set them to 0.2 (see Figure 4.9).

   We generate masks $m_s$ such that

$$m_s(t) = \begin{cases} \mathrm{mean}(M) \times f(t) + 0.2\,, & \text{if slope is positive,} \\ -\mathrm{mean}(M) \times f(t) + 1\,, & \text{if slope is negative,} \\ 1, & \text{if slope is 0.} \end{cases} \tag{4.1}$$

   The function $f(t)$ is a linear discrete increase in duration $d$ such that f(t) = 1:1:d (in MATLAB notation[1]) and $d = \frac{0.8}{\mathrm{mean}(M)}$ is the duration of increase from 0.2

[1] An example sequence for the MATLAB notation f(t) = 1:1:d is $1, 2, 3, 4, ...$

to 1 given the mean gain mean($M$). The masks that we use in our study are symmetric along their maxima, so the increasing and decreasing slopes have equal absolute values. (See Figure 4.3c for an illustration of amplitude masks.) We start a new envelope at the beginning of each pulse train.

4. Mask the constant-pulse audio track $S_c(t)$ with gain functions $m_s(t)$ and thereby generate a masked audio track $S_m(t) = S_c(t) \times m_s(t)$.

The only difference between playback signals to a species are the amplitude envelopes; other features of recorded song are unchanged (see Figure 4.8). Following other authors,[54,56,58] we measure amplitude as the maximum of the pulses. An alternative measure of amplitude is peak-to-peak amplitude, which is the difference between the maximum and minimum peak of a pulse and represents the maximum signal that a female can detect.[31] To test whether the choice of measuring maximum amplitude versus peak-to-peak amplitude makes a significant difference, we examine the Pearson correlation of the two measures in our song recordings and find that they are significantly positively correlated ($R^2 \approx 0.96$, with a p-value of $P < 0.0001$), which suggests that maximum amplitude and peak-to-peak amplitude are comparable.

We control for the audibility of pulses in modulated song, ensuring that flies can hear small amplitude pulses in playbacks (see Figure 4.9). To test whether flies can hear pulses with minimum amplitude in songs, we generate an artificial song in which we set all pulses to the minimum amplitude and play it back to flies. If pulses are too low in amplitude for flies to hear, flies would respond to small-amplitude pulses in a similar way as to a song that lacks pulses altogether. In contrast, we find that flies respond significantly more to a song with small-amplitude pulses than to song that lacks pulses (see Figure S4.9c), which demonstrates that small-amplitude pulses in our playbacks are processed by flies. We observe that flies do not respond to songs that lack pulses even when they contain sine song. This agrees with previous findings that showed that flies do not chain in response to sine song.[66]

## 4.3 Results

### 4.3.1 *D. melanogaster* and *D. simulans* females prefer the SAS of their species

The sibling species *D. melanogaster* and *D. simulans* differ in their songs, producing different frequencies, pulse shapes, and IPI (see Figure 4.9). We examine whether

the two species also differ in SAS, which we introduce in Chapter 3, and we thus measure the SAS of *D. melanogaster* and *D. simulans* males. (See Section 3.2 for a mathematical definition of SAS.) See Figure 4.1a for an illustration of measuring and visualizing SAS in song. *D. melanogaster* and *D. simulans* differ in their SAS. SAS gain is the relative increase in amplitude between successive pulses (see Figure 4.1b), and we suggested in Chapter 3 that SAS gain may be a correlate of muscle contraction. *D. simulans* males produce songs with steeper SAS gain than *D. melanogaster* males (see Figure 4.1c; $P < 0.0001$; WR).

We further investigate the structure of SAS in the two species. See Figure 4.2a and Figure 4.1e for an illustration of SAS in *D. melanogaster* and *D. simulans* songs, respectively; and see Table 4.1 for species' mean SAS gain, amplitude and duration. SAS gain is variable and the majority of observed SAS gain is flat (low normalised amplitude per second). This is true for both *D. melanogaster* and *D. simulans*. We find that the gain of *simulans* SAS is significantly steeper than of *melanogaster* SAS (see Figure 4.1c). However dot plots of SAS of the two species do not reflect this finding. Dot plots of both *melanogaster* SAS (Figures 4.2b-d) and *simulans* SAS (Figures 4.2f-h) are noisy.

One can test female preference for song characteristics by deafening males and thereby making them unreceptive to song playback.[35] For our copulation assays, we deafen males by removing their arista. Because deafened males do not respond to song playback,[35,66,70] we interpret our results for copulation assays in terms of female auditory responses and mating decisions.

We find that *D. melanogaster* and *D. simulans* song differ in their SAS, so we examine whether female flies make mating decisions based on SAS differences, analogous to females making mating decisions based on IPI differences.[29,36,70] (See Section 4.2.4 for a description of how we modify SAS in our playback stimuli.) To test this, we use copulation frequency in response to playback as a behavioural assay.[29,36,70] (For an illustration of the copulation assay see Figure 4.3b.) We develop a method of playback-stimulus design in which we manipulate pulse amplitude envelopes in recordings of courtship song to generate realistic stimuli (see Figure 4.3c and Section 4.2.4) that are more effective than artificial stimuli at eliciting flies' auditory responses in our playback setup (see Figure 4.10).

Female *D. melanogaster* rarely copulate with wing-cut males that cannot sing (the 'silence condition'; see Figures 4.5a and 4.5b). However, females copulate with muted males when they are exposed to playback with species-specific characteristics.[29,36] In agreement with this finding, we observe that *D. melanogaster* females copulate

significantly more to *D. melanogaster* songs masked with mean gain envelopes of *D. melanogaster* (m-m) than in response to *D. melanogaster* songs masked with *D. simulans* mean gain envelopes (m-s) or silence. (The p-value is $P < 0.001$, WR; see Figure 4.5a.) Female *D. melanogaster* copulate at low levels both during silence and during playback of songs masked with *D. simulans* mean gain envelopes (m-s). Interestingly, copulation levels do not differ between silence and playback of songs masked with *D. simulans* mean gain envelopes (m-s) ($P \approx 0.78$, WR), suggesting that *D. melanogaster* song is rendered unattractive to females by applying envelopes with *D. simulans* mean gain. Notably, other song parameters — such as IPI, pulse shape, pulse frequency, and sine frequency — are indistinguishable between playback conditions (see Figure 4.8), suggesting that a female's copulation choice is affected strongly by the SAS envelope ($P < 0.001$; $F \approx 38.03$ in a one-way anova; and $n_c = 177$, where $n_c$ is the number of couples with one male and one female).

Consistent with our observations in the above paragraph, female *D. simulans* copulate more to *D. simulans* song masked with *D. simulans* mean gain envelope (s-s) than to silence control and *D. simulans* song masked with *D. melanogaster* mean gain envelope (s-m) ($P < 0.005$, WR). *D. simulans* females copulate at low levels both during silence and during playback with *D. melanogaster* envelopes ($P \approx 0.92$, WR; see Figures 4.4b and 4.5b). SAS affects the fraction of copulating pairs in *D. simulans* ($P < 0.001$, $F \approx 26.78$ in a one-way anova, and $n_c = 246$), which shows that the closely related species *D. melanogaster* and *D. simulans* prefer their own species' envelope, suggesting that SAS is a communication signal in *Drosophila*.

In agreement with previous findings that *D. melanogaster* are tuned to their species song characteristics,[15,29,66,70] we find that *D. simulans* song is unattractive to *D. melanogaster* (see Figure 4.4c and 4.5c). We find that *D. melanogaster* copulate at similar levels in the silence control conditions presented in panels 4.4a and c ($P \geq 0.05$, WR). This implies that *D. melanogaster* behaved comparably across experiments.

We examined whether *D. simulans* couples copulate in response to *D. melanogaster* song, masked with *D. simulans* or *D. melanogaster* SAS, and find that *D. simulans* increase copulation rates relative to silence (see Figures 4.4d and 4.5d), which suggests that *D. melanogaster* song has some stimulatory effect on *D. simulans*. This effect may be due to *D. melanogaster* song being similar to *D. simulans* song (see Figure 4.8). Additionally *D. simulans* couples seem to be more prone to copulation than *D. melanogaster* couples; the mean copulation level during silence of *D. simulans* couples is higher than of *D. melanogaster* couples. Importantly, *D. simulans* couples do not

behaviourally discriminate between *D. melanogaster* song masked with *D. simulans* SAS and *D. melanogaster* song masked with *D. melanogaster* SAS (see Figure 4.4d and 4.5d), which suggests that flies use SAS in combination with other song characteristics (such as their species song frequency and IPI) to make mating choices. This agrees with how other song characteristics seem to affect mating choice.[121] We find that *D. simulans* copulate at significantly different levels in the silence control conditions presented in Figures 4.4b and 4.4d ($P \leq 0.001$, WR). This implies that *D. simulans* behaved differently across experiments. One reason for this observation could be differences in female age in the two experiments: in panel 4.4b we present data from females after ageing them for seven days and in 4.4d we show data of different females after ageing them for four days. Younger *D. simulans* females have a lower baseline copulation level than older females, which is consistent with observations of other researchers[122] and our two experiments may not be comparable (see Figures 4.4b and 4.4d).

### 4.3.2 *D. melanogaster* females differentiate SAS

*D. melanogaster* females can differentiate song parameters, such as IPI, preferring their own species' IPI over shorter or longer IPI durations[29,70] (see Figure 4.6a). We find that *D. melanogaster* females prefer their species SAS over their sibling species *D. simulans*'s SAS, so we examine whether *D. melanogaster* females can differentiate their species SAS gain over both flatter SAS and steeper SAS gain. For testing female SAS preference, we designed an experiment with four playback conditions: (1) song with constant amplitudes (flat gain; see Figure 4.6b), (2) song with *D. melanogaster* SAS mean gain envelope (m-m; see Figure 4.6b), (3) song with double the *D. melanogaster* SAS mean gain envelope (m-2m; see Figure 4.6b), and (4) a silence control condition in which we do not play back song to male and female flies. We find that *D. melanogaster* females prefer their species SAS gain over both flatter gain and steeper gain (the p-value is $P < 0.001$, WR; see Figure 4.6b), suggesting that female *D. melanogaster* prefer their species SAS gain both over flatter and steeper SAS gain.

Other playback studies use artificial song for copulation assays,[29,70] and we thus examine whether *D. melanogaster* females can differentiate SAS when it is applied to artificial *D. melanogaster* song. For testing this, we designed an experiment analogous to the one described in the paragraph above, with the only difference being that we apply SAS gain envelopes to artificial *D. melanogaster* song rather than to song

recordings of *D. melanogaster* males. We again find that females prefer their species SAS mean gain over flatter and steeper gain (the p-value is $P < 0.001$, WR; see Figure 4.6c), suggesting that females robustly show a bandpass-like preference for their species SAS.

### 4.3.3 *D. melanogaster* and *D. simulans* males differentiate SAS

One can test male hearing behaviourally using a chaining paradigm, in which males start to court each other and form courtship chains in response to song playback.[99,100] Similar to females, male flies are stimulated more by songs with their own species' characteristics and chain more in response to songs with their species IPI.[66,72] We thus examine whether male flies also differentiate SAS, and we assess their chaining levels in response to songs with different SAS to test this. (See Figure 4.7a for an illustration of the chaining assay.)

We find that *D. melanogaster* males chain significantly more in response to *D. melanogaster* song masked with *D. melanogaster* mean gain envelope than in response to *D. melanogaster* song masked with *D. simulans* mean gain envelope [see Figure 4.7b; the p-value is $P < 0.001$, WR], which is consistent with female preferences in the copulation assay (see Figure 4.4a and 4.5a). Additionally, *D. melanogaster* males chain more in response to their species SAS mean gain envelope than in response to flatter and steeper SAS gain envelopes ($P < 0.001$; WR; see Figure 4.7b). Interestingly, *D. simulans* males show bandpass-like increase in chaining for gain, as they chain more in response to flat gain and *D. simulans* gain envelopes than in response to envelopes that are between these extremes (see Figure 4.7c). Similarly, *D. simulans* males chain more in response to *D. simulans* gain envelope and the steepest gain envelope (m-3m) than in response to gain envelopes that are between these extremes ($P < 0.05$, WR; see Figure 4.7c). Our results demonstrate that both female and male flies differentiate SAS envelopes and that they exhibit bandpass-like preferences for SAS gain.

## 4.4 Discussion

Our work demonstrates that flies respond behaviourally to SAS (as introduced in Chapter 3) in their courtship song, suggesting that *Drosophila* can auditory process SAS. Our study thereby establishes processing of SAS as an area of auditory research

in *Drosophila*. We make our MATLAB code for manipulating gain in courtship song of *Drosophila* publicly available;[120] this code can be used to modulate SAS in courtship song recordings and in artificial song.

Notably, SAS can extend over long timescales (e.g., hundreds of milliseconds; see Table 4.1), which may help explain why female flies have been reported to process auditory information about male courtship song over similarly long timescales.[117] Future work could investigate a potential link between long-timescale auditory processing and SAS.

Our work shows that *D. melanogaster* behaviourally discriminate songs with differently-shaped amplitude masks. The masks that we apply in our current study are diamond-like, and our work shows that flies can discriminate these songs from songs with constant-amplitude rectangular-like masks, which suggests that flies may be able to process differences in the shape of SAS masks. It will be interesting to test whether flies discriminate different shapes of SAS masks in future work.

In this Chapter we have reported amplitude modulations in pulse song and others have reported them also.[31,51] However do amplitude modulations exist in sine song? The study of sine song is difficult because it has low mean intensity[26,51] and no reliable automated methods exist to detect sine song.[31,32] Therefore, a comprehensive analysis of sine song amplitude modulation will require improvements both in recording techniques and song analysis software.

We find that SAS gain differs in the closely related species *D. melanogaster* and *D. simulans* and that females prefer their own species' SAS gain, which suggests that SAS gain is an ethologically meaningful signal. SAS gain may be linked to muscle contraction, although this is difficult to verify experimentally as we discuss in Chapter 3 and in Chapter 7. However, it is likely that neuromuscular differences in the song-generating system of *D. melanogaster* and *D. simulans* contribute to differences in SAS.

To our knowledge a comparative analysis of the differences between muscles and neurons that control courtship song in *D. melanogaster* and *D. simulans* does not exist. However, muscles and neurons have been studied extensively in *D. melanogaster* using both physiological[48,51] and neurogenetic tools.[30,37,38] CRISPR-Cas9 technology[123] makes it possible to apply neurogenetic tools that had been available only for *D. melanogaster* to other *Drosophila* species such as *D. simulans*, which in turn makes it possible, as a long-term goal, to compare the song-generating circuits and muscles in *D. melanogaster* and *D. simulans*.

Our work shows that SAS is a communication signal and flies respond to SAS similarly as they respond to IPI. IPI is an established signal in the study of *Drosophila* song, and flies communicate species type with IPI.[29,36,66] Further work is needed to ascertain a potential role of SAS in species communication. From studying IPI, we know that flies possess auditory circuits that act as bandpass filters for their species IPI.[70–72] It will be interesting to examine whether SAS can be processed analogously.

We find that *Melanogaster* females and males show similar preferences for SAS—both sexes prefer their species SAS gain over steeper or flatter gains. This might suggest that males and females process SAS similarly. Importantly however, we test behavioural preference of SAS with distinct paradigms in the two sexes: We use copulation to assess female auditory response and chaining to assess male responses. Copulation and chaining are different behaviours and can not be directly compared. Thus our findings do no prove that males and females respond similarly to SAS. In addition chaining has no obvious evolutionary meaning (see Section 4.1 for a discussion), which makes it difficult to deduce what different levels of chaining mean. One approach to overcome the restrictions of the chaining paradigm could be to assess copulation in males. So far we have measured copulation when playing back song to females and males, which we muted and deafened. Thus we focused on female responses, as males were not able to hear our playback. However researchers could play back song to couples of females and males with intact ears, thus approximating the male response. It would be possible to deafen females also, thereby trying to parse out male responses independent from female ones. Note that we show that deaf males copulate with females, which suggests that males may not require auditory stimulation to copulate.[66] In contrast females seem to require auditory stimulation to copulate.[26,29,36] Therefore it needs to be tested if the copulation assay is sensitive for studying male auditory responses.

We measure SAS gain, duration and amplitude and show that *Drosophila* can behaviourally discriminate songs masked with SAS gain envelopes. It may be possible to measure SAS also using spectral analysis. This analysis has been used to study Kyriacou-and-Hall (KH) cycles that describe variations in IPI duration.[124] Recently however, spectral analysis for the study of KH cycles yielded contradictory conclusions, failing to detect KH cycles[119] or detecting KH cycles[33]—which suggests that the methods that have been employed for spectral analysis of song fail to reliably detect patterns in *Drosophila* song. Spectral analysis detects regular patterns[119] and song data are noisy[31] and sensitive to experimental procedures like fly husbandry.[33]

It is not obvious what methods to use to analyse patterns in noisy biological data, and the fly song field may profit from further methodological development.[32,33,119,124]

We find that SAS in both *melanogaster* and *simulans* song is noisy (see Figure 4.2). The structure of SAS should be further examined to elucidate the presumed structure of SAS. Spectral analysis could be a method to investigate SAS, however spectral analysis assumes regular patterns, and SAS may not follow regular patterns.

| Song parameters | *D. melanogaster* ($n_s = 16$) | *D. simulans* ($n_s = 17$) |
|---|---|---|
| Pulse song frequency in Hz (SE)* | 196.2 (2.7) | 298.8 (5.9) |
| Sine song frequency in Hz (SE)* | 173.3 (2.2) | 167.7 (2.5) |
| Mean IPI** in ms (SE)* | 34.0 (0.4) | 49.6 (1.0) |
| Cycles per pulse (SE)* | 1.9 (0.08) | 1.6 (0.04) |
| Wing extension index*** | 0.79 | 0.75 |
| SAS gain in normalized amplitude per second (SE)* | 1.50 (0.10) | 1.92 (0.13) |
| Normalized amplitude (SE)* | 0.20 (0.012) | 0.25 (0.016) |
| SAS duration in ms (SE)* | 127.1 (2.9) | 134.9 (3.8) |

**Table 4.1:** Song statistics and wing extension index (WEI) of recordings for *D. melanogaster* and *D. simulans*

* SE is the standard error of the mean, and $n_s$ indicates the number of flies we used. ** IPI is the inter-pulse interval. *** The wing extension index (WEI) is the fraction of time that a male fly spends extending one wing during recording. We give the WEI of one representative 10-minute recording.

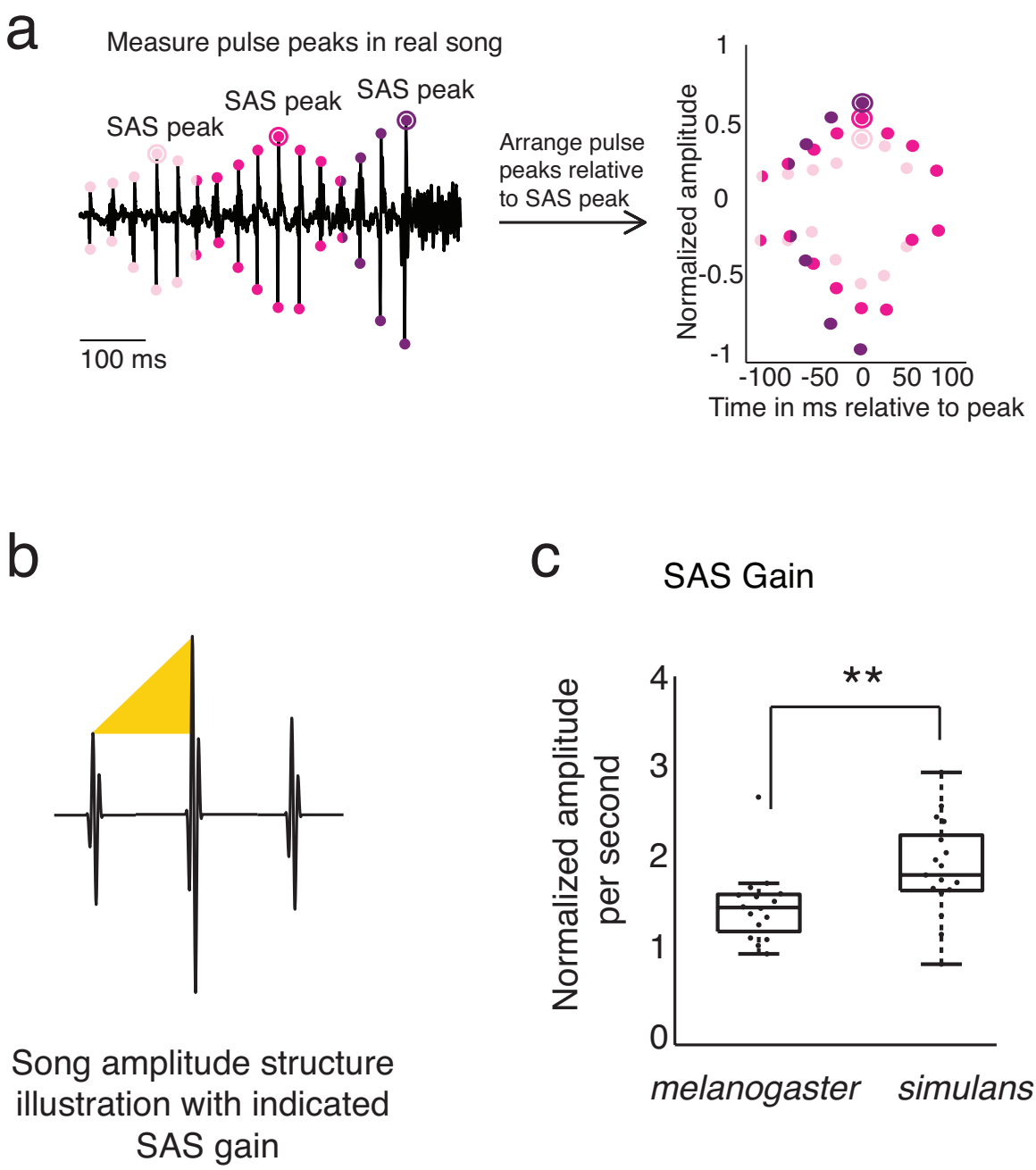



**Figure 4.1:** SAS differs in *D. melanogaster* and *D. simulans*.
(**a**) Workflow for visualizing SAS. We first (1, left panel) define SAS as amplitude increase across a local amplitude peak (circled dots) followed by a decrease to a local minimum (semicircle). We then (2, left panel) detect pulse maxima and minima (dots) in courtship song recordings (in *D. melanogaster* for this example). We detect three SASs in the depicted pulse train, and we mark them with different dot colors (light pink, dark pink, and purple). (**b**) Illustration of SAS gain as a yellow triangle on a song trace. SAS gain is the relative increase in amplitude over time, measured as the difference in amplitude between two successive pulses. (**c**) Comparison of SAS gain between *D. melanogaster* (with $n_s = 16$ flies) and *D. simulans* (with $n_s = 17$ flies). See Table 4.1 for the mean SAS gain values per species. We overlay box plots with bee plots that show the data distributions. Each dot represents the mean parameter value of an individual fly. The asterisks indicate significance levels: ** indicates a p-value of $P < 0.005$ (after Bonferroni correction for multiple testing using WR).

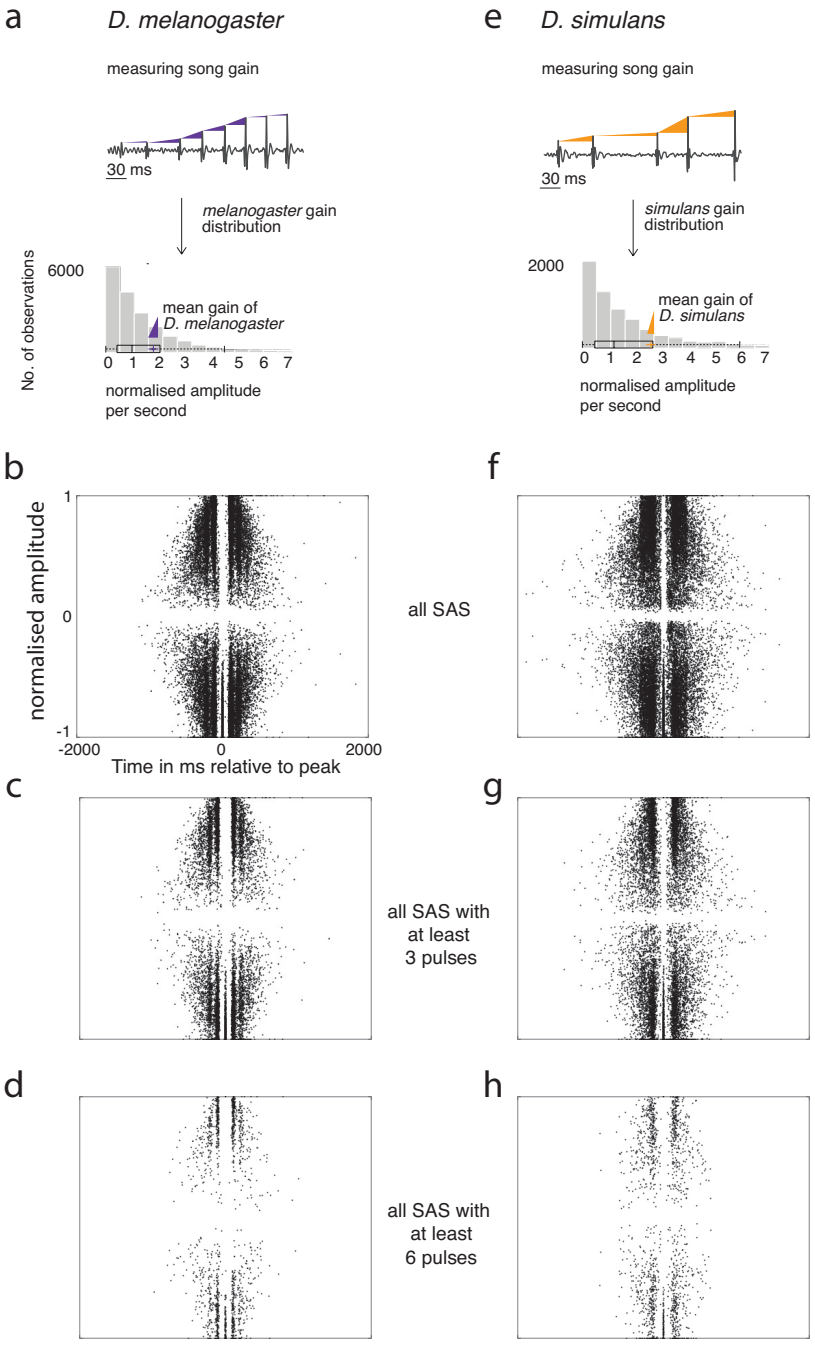



**Figure 4.2:** What is the structure of SAS in *D. melanogaster* and *D. simulans*?
(**a**) Measuring SAS gain in song of *D. melanogaster*. The distribution shows variable SAS gain: Most observed gains are flat (low normalised amplitude per second). (**b**) Dot plot of pulse amplitudes in *D. melanogaster* for all SAS (with $n_p = 51500$ pulses). We generated dot plots similarly as shown in Figure 4.1. In contrast to Figure 4.1 we normalised SAS with SAS peaks, instead of the absolute peak across songs. Thus each SAS peak was transformed to 1. (**c**) Dot plot of pulse amplitudes in *D. melanogaster* for all SAS with at least 3 pulses (with $n_p = 14664$ pulses). (**d**) Dot plot of pulse amplitudes in *D. melanogaster* for all SAS with at least 6 pulses (with $n_p = 2032$ pulses). (**e**) Measuring SAS gain in song of *D. simulans*. The distribution shows variable SAS gain. (**f**) Dot plot of pulse amplitudes in *D. simulans* for all SAS (with $n_p = 51500$ pulses). (**g**) Dot plot of pulse amplitudes in *D. simulans* for all SAS with at least 3 pulses (with $n_p = 14664$ pulses). (**h**) Dot plot of pulse amplitudes in *D. simulans* for all SAS with at least 6 pulses (with $n_p = 2032$ pulses).

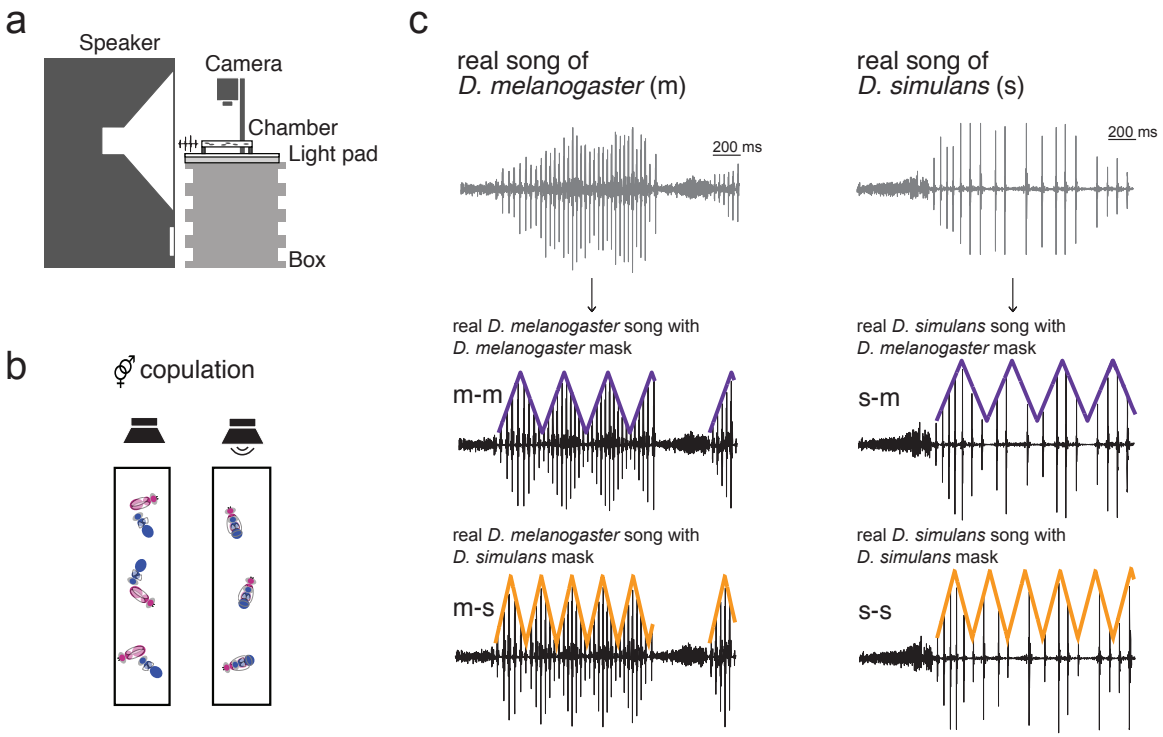



**Figure 4.3:** Experimental setup and stimuli design for testing auditory responses to SAS in *Drosophila*.

(**a**) Schematic of playback setup. (**b**) Schematic of copulation assay. (**c**) We create playback stimuli by masking five minutes of species-specific real song with species' mean gain envelopes. Songs of *D. melanogaster* and *D. simulans* exhibit species-specific differences (see Table 4.1), and our amplitude modulation does not alter song parameters (see Figure 4.8). We use the following abbreviation scheme for our playback conditions: m-m refers to *D. melanogaster* song with *D. melanogaster* mean gain, m-s refers to *D. melanogaster* song with *D. simulans* mean gain, and so on.

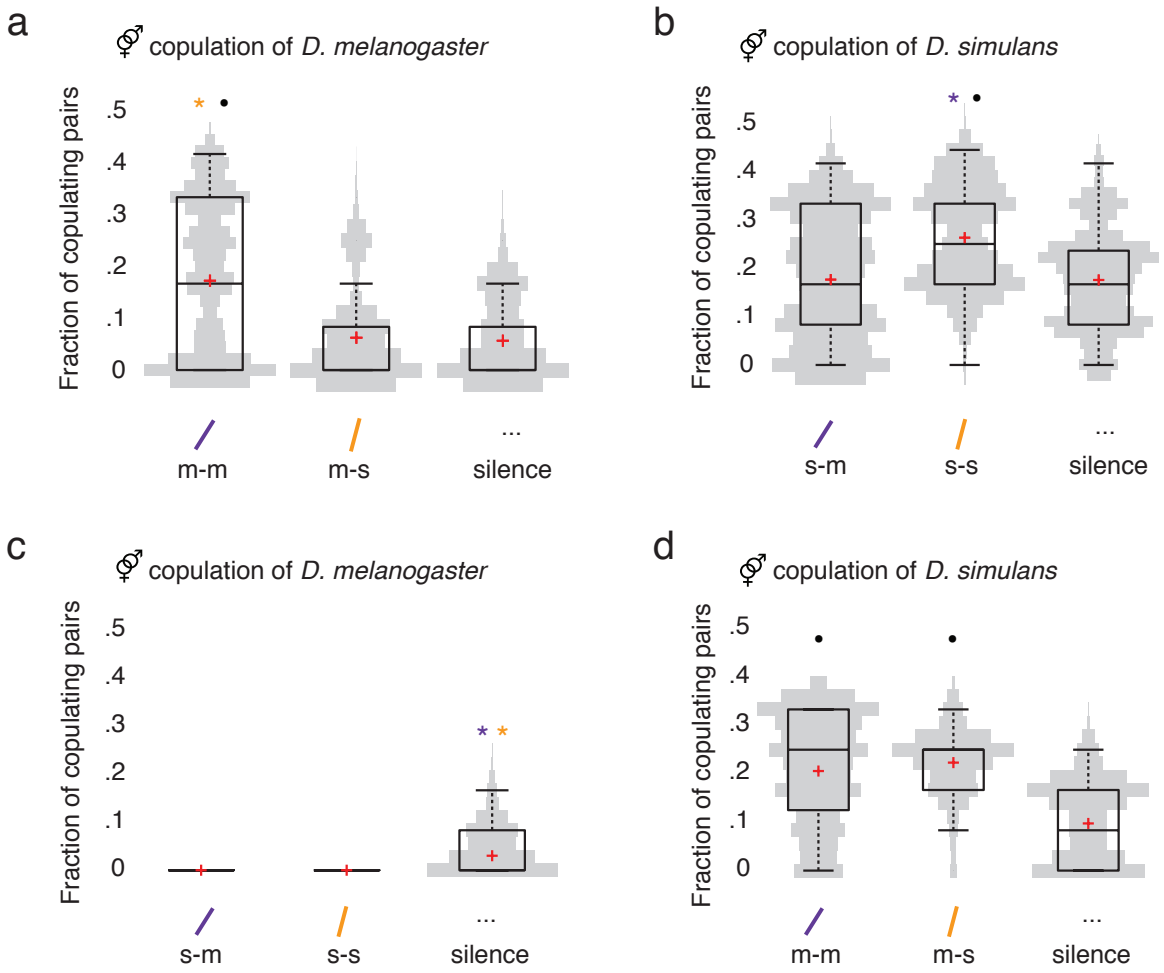



**Figure 4.4:** Female *D. melanogaster* and *D. simulans* differentiate between each others' SAS envelope.

(**a**) Fraction of *D. melanogaster* couples copulating during a 20-minute song playback and during a 20-minute silence control. (**b**) Copulation fraction of *D. simulans* couples over 20-minute playback and silence control. We show the distribution of fraction of copulating pairs as grey kernel density plots, which we mirror across the vertical axis and as box plots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the inter-quartile-range (IQR), and the whiskers indicates 1.5 × IQR. (**c**) Copulation fraction of *D. melanogaster* couples over 20-minute playback and silence control. *D. melanogaster* couples do not copulate in response to *D. simulans* song masked with either *D. melanogaster* or *D. simulans* SAS ($n_c = 172$ mixed-gender couples). (**d**) Copulation fraction of *D. simulans* couples over 20-minute playback and silence control. *D. simulans* couples copulate at similar rates in response to *D. melanogaster* song masked with either *D. simulans* SAS or *D. melanogaster* SAS ($n_c = 180$ mixed-gender couples). We show the distribution of fraction of copulating pairs as grey kernel density plots, which we mirror across the vertical axis and as box plots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 × IQR. The asterisks indicate that there is a significant difference in fraction of copulating pairs (a p-value of $P < 0.01$, WR). The color of the asterisk indicates which gain condition differs significantly from the others. For example, the orange asterisk signifies that the orange condition (*D. simulans* mean gain envelope) differs significantly from the labeled condition (*D. melanogaster* mean gain envelope). The black dot indicates a significant difference in the fraction of copulating pairs between the labeled condition and silence control (a p-value of $P < 0.01$, WR).

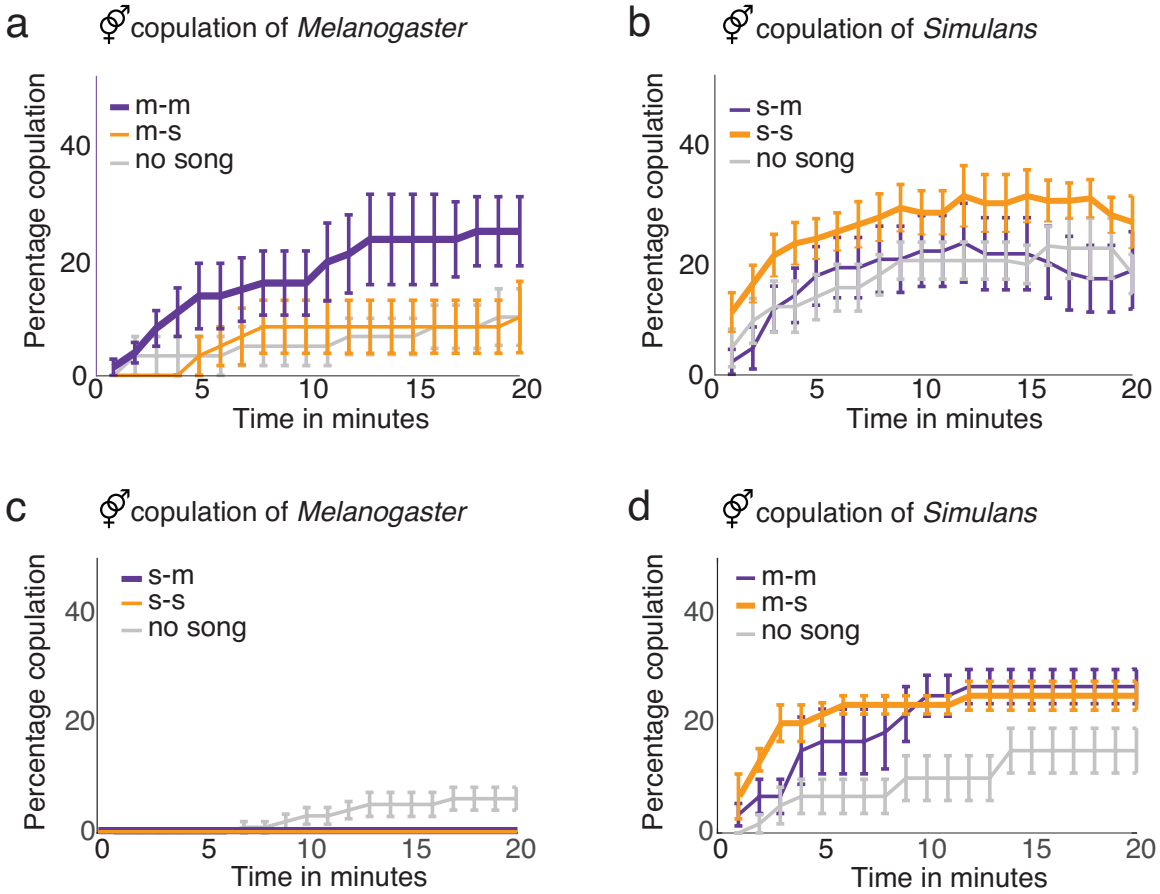



**Figure 4.5:** Female *D. melanogaster* and *D. simulans* differentiate between each others' SAS envelope.

Data from Figure 4.5 presented as percentages over time. (**a**) Percentage of *D. melanogaster* couples copulating per minute over a 20-minute *Melanogaster* song playback and over a 20-minute silence control. (**b**) Percentage of copulation of *D. simulans* couples per minute over 20-minute *Simulans* song playback and silence control. (**c**) Percentage of *D. melanogaster* couples copulating per minute over a 20-minute *Simulans* song playback and over a 20-minute silence control. (**d**) Percentage of copulation of *D. simulans* couples per minute over 20-minute *Melanogaster* song playback and silence control.

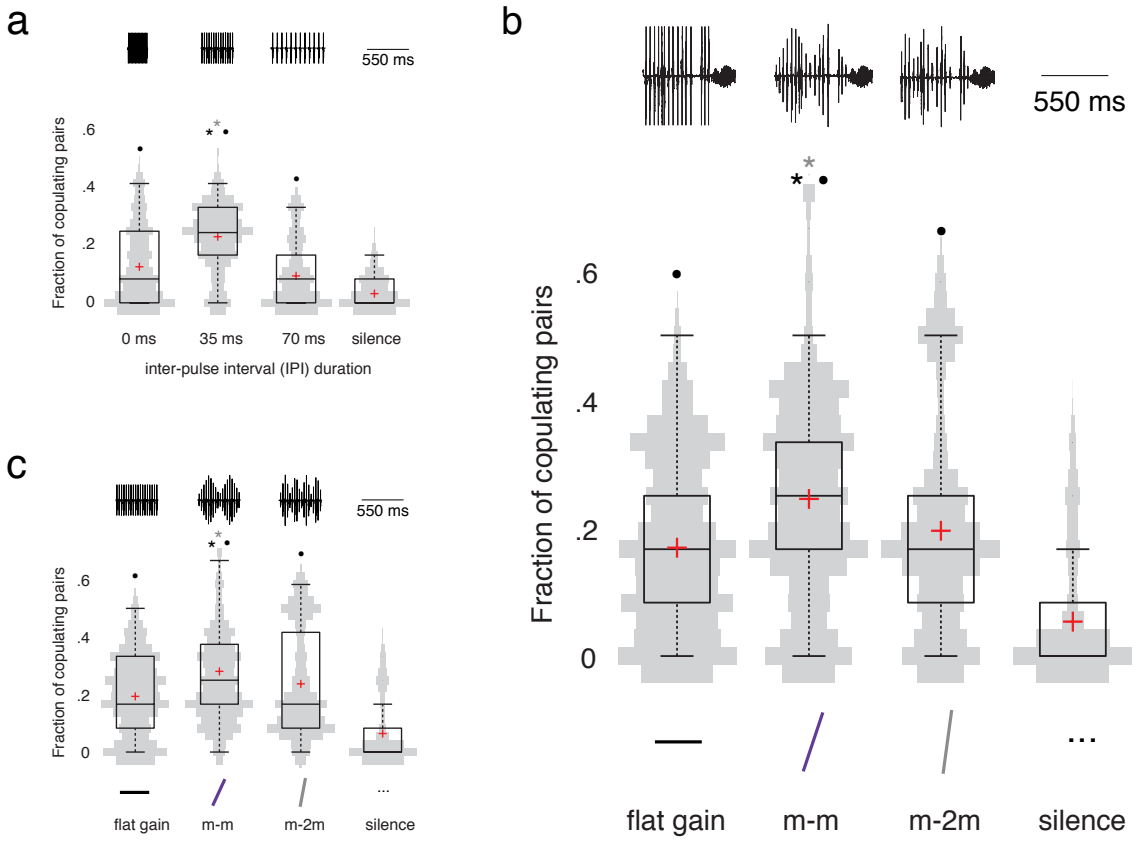



**Figure 4.6:** SAS envelope preference of female *D. melanogaster*.
(**a**) *D. melanogaster* females prefer their species IPI duration (IPI = 35 ms) over shorter IPI (0 ms), longer IPI (70 ms), and the silence control condition ($P < 0.0001$, $F \approx 70.57$ in a one-way anova, $n_c = 264$ mixed-gender couples). (**b**) *D. melanogaster* females prefer their species SAS envelope (m-m) over flat gain, steeper gain (m-2m), and the silence control condition ($P < 0.0001$; $F \approx 55.63$ in a one-way anova test; and $n_c = 444$, where $n_c$ is the number of couples with one male and one female) when SAS is modulated in a courtship song recording of a *D. melanogaster* male. (See Figure 4.3b for an illustration of SAS modulation in courtship song recordings.) (**c**) *D. melanogaster* females prefer their species SAS envelope (m-m) over flat gain, steeper gain (two times the *D. melanogaster* mean gain; m-2m), and the silence control condition ($P < 0.0001$, $F \approx 38.74$ in a one-way anova, $n_c = 228$ mixed-gender couples) when SAS is modulated in an artificial *D. melanogaster* song. We show the distribution of the fraction of copulating pairs as grey kernel density plots, which we mirror across the vertical axis and as box plots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 × IQR. The asterisks indicate a significant difference in the fraction of copulating pairs (a p-value of $P < 0.01$, WR). The color of the asterisk indicates which gain condition differs significantly from the others. For example, the grey asterisk signifies that the grey condition (m-2m envelope) differs significantly from the labeled condition (*D. melanogaster* mean gain envelope). The black dot indicates a significant difference in the fraction of copulating pairs between the labeled condition and the silence control (a p-value of $P < 0.01$, WR).

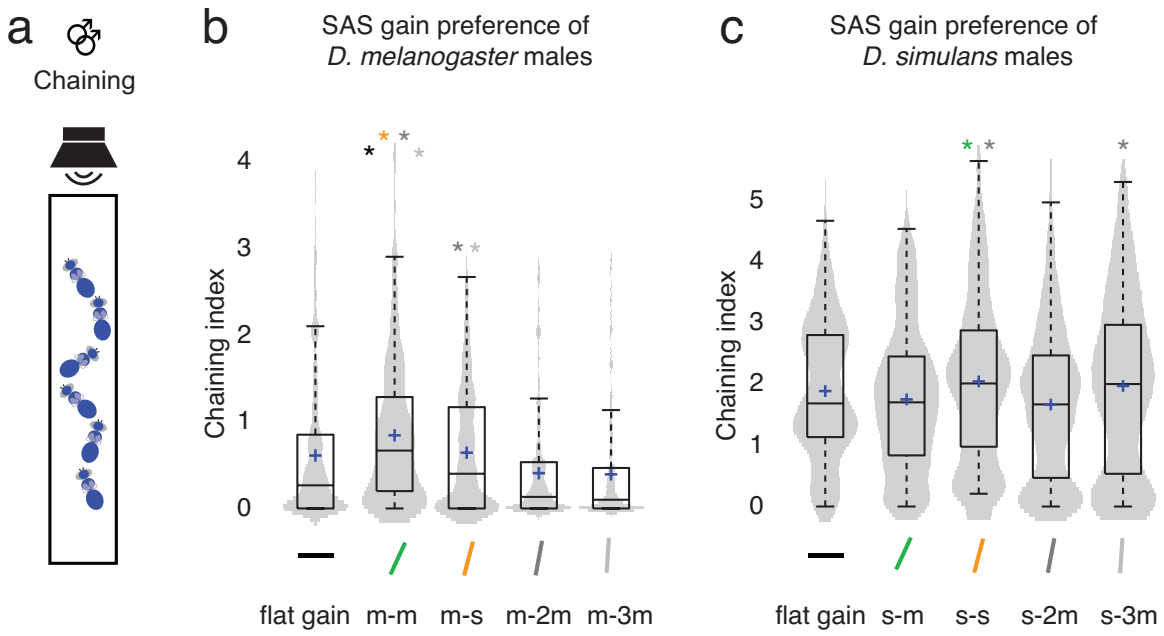



**Figure 4.7:** SAS envelope preference of male *D. melanogaster* and male *D. simulans*.
(**a**) Schematic of chaining assay. ChaIN,[66] the automated tracking software that we use to assess chaining, counts the number of males in a chain at a given second (i.e., the chaining index[66]). There are 6 males per group, so the maximum number of chaining males is 6 (and the minimum is 0). (**b**) *D. melanogaster* males chain most in response to *D. melanogaster* mean gain. We show the distribution of chaining during a 5-minute playback of *D. melanogaster* song masked with a range of gains. We illustrate our results using colored bars below the plots as grey kernel-density plots, which we mirror across the vertical axis and as box plots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicate 1.5 × IQR. The asterisks indicate a significant difference in chaining (a p-value of $P < 0.01$, WR). The color of the asterisk indicates which gain condition differs significantly from the others. For example, the orange asterisk signifies that the orange condition (*D. simulans* mean gain envelope) differs significantly from the labeled condition (*D. melanogaster* mean gain envelope). To avoid redundancy, we indicate significant differences between gain conditions only at the larger of the two gain conditions. The playback conditions are *D. melanogaster* song masked with (1) flat gain with constant amplitude ($n_g = 20$, where $n_g$ is the number of male groups, with six males per group), (2) *D. melanogaster* mean gain envelope (m-m; $n_g = 20$ groups), (3) *D. simulans* mean gain envelope (m-s; $n_g = 20$ groups), (4) two times the *D. melanogaster* mean gain envelope (m-2m; $n_g = 15$ groups), and (5) three times the *D. melanogaster* mean gain envelope (m-3m; $n_g = 16$ groyps). (**c**) *D. simulans* exhibit a bandpass-filter-like preference for song masked with *D. simulans* mean gain envelopes. The asterisks indicate a significant difference in chaining (a p-value of $P < 0.01$, WR). The playback conditions are *D. simulans* song masked with (1) flat gain with constant amplitude ($n_g = 17$ groups), (2) *D. melanogaster* mean gain envelope (s-m; $n_g = 18$ groups), (3) *D. simulans* mean gain envelope (s-s; $n_g = 24$ groups), (4) two times the *D. melanogaster* mean gain envelope (s-2m; $n_g = 25$ groups), and (5) three times the *D. melanogaster* mean gain envelope (s-3m; $n_g = 19$ groups).

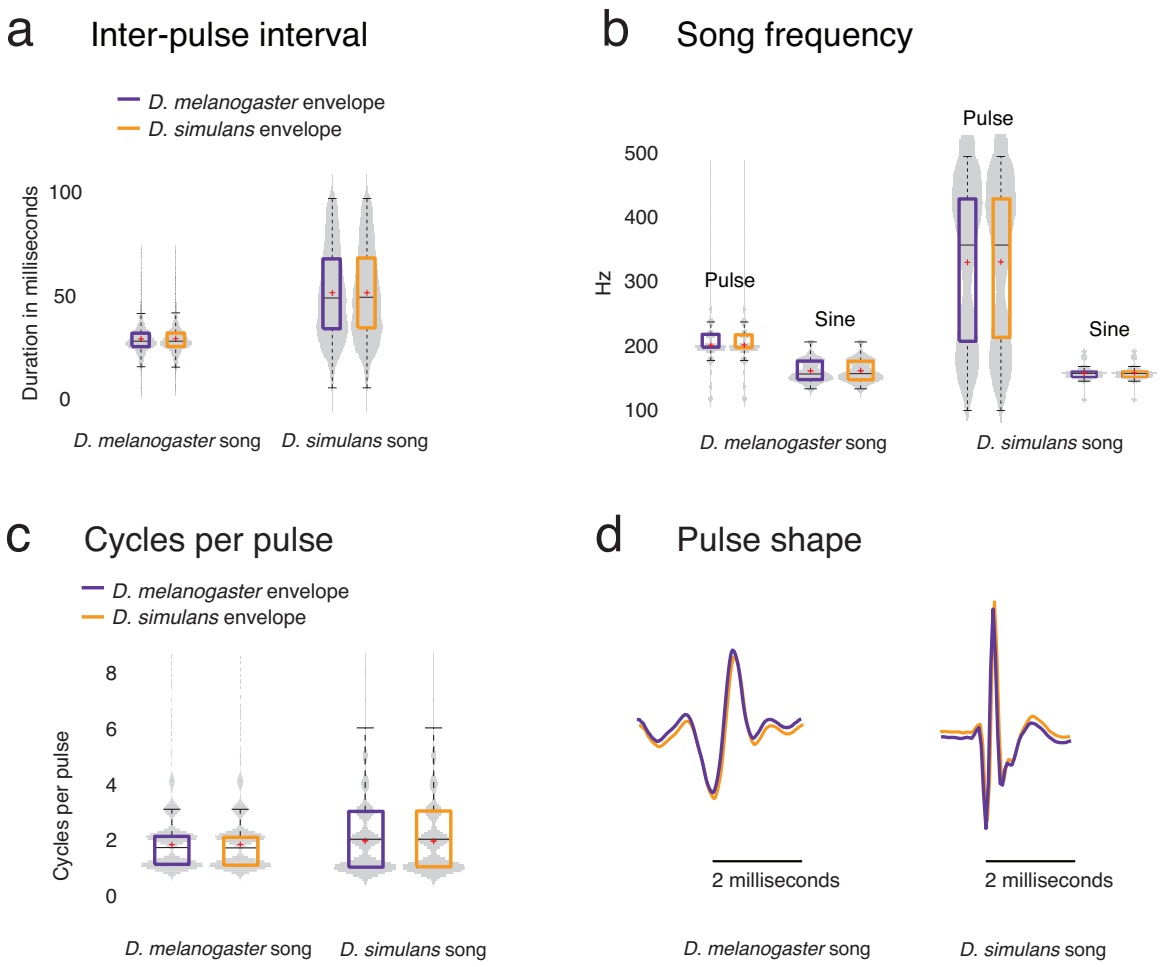



**Figure 4.8:** Song parameters do not differ significantly across playback conditions.
(**a**) IPI does not differ significantly across different playback conditions. To measure IPI, we detect pulses in modulated playbacks automatically with FlySongSegmenter[32] and then compute the distance between detected pulses. The number $n_P$ of detected pulses does not differ significantly across different playback conditions for *D. melanogaster*; *D. melanogaster* song has $n_P = 1873$ for m-m, and $n_P = 1869$ for m-s, where m-m refers to *D. melanogaster* song with *D. melanogaster* mean gain, m-s refers to *D. melanogaster* song with *D. simulans* mean gain. Number $n_P$ also does not differ significantly across playback conditions for *D. simulans*. (The $n_P = 827$ for s-m and $n_P = 817$ for s-s, where s-m refers to *D. simulans* song with *D. melanogaster* mean gain, s-s refers to *D. simulans* song with *D. simulans* mean gain.) We show song statistics per species (*D. melanogaster* song, and *D. simulans* song song) and per species envelope condition (green for *D. melanogaster* envelope, and orange for *D. simulans* envelope) as grey kernel-density plots, which we mirror across the vertical axis and as box plots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the inter-quartile-range (IQR), and the whiskers indicates 1.5 × IQR. (**b**) Pulse song frequency does not differ significantly across different playback conditions for each tested species. (**b**) Sine song frequency does not differ significantly across playback conditions for each tested species. (**d**) Mean pulse shape (computed as a pointwise mean) and cycles per pulse do not differ significantly across different playback conditions for each tested species.

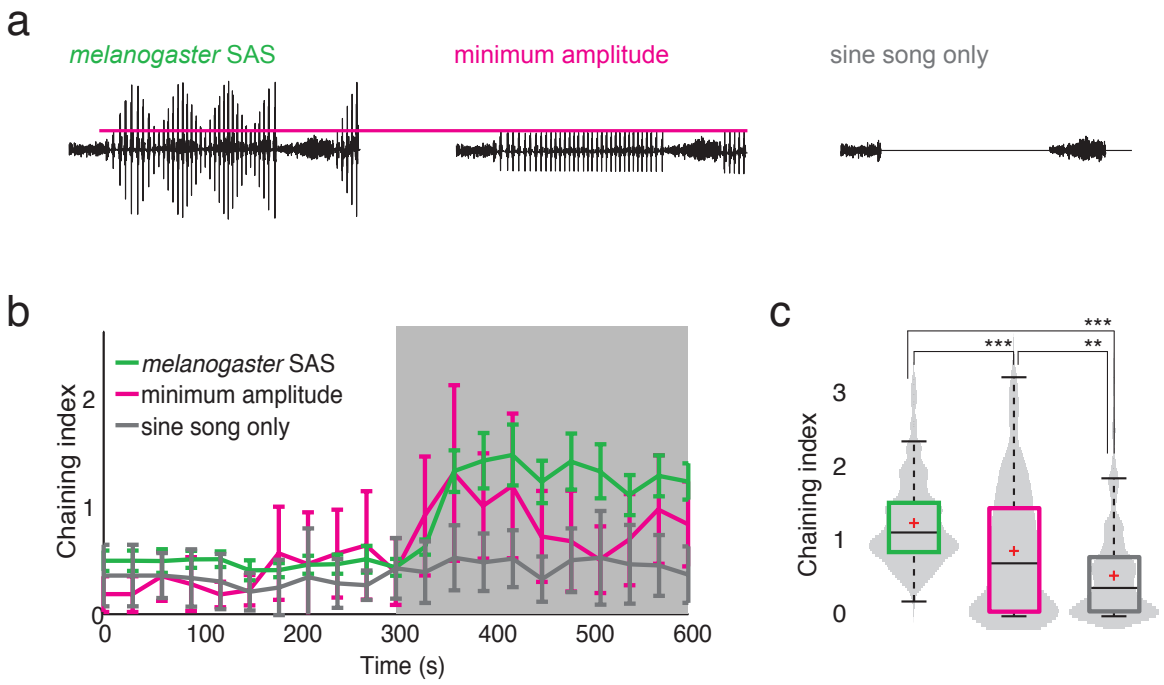



**Figure 4.9:** Small-amplitude pulses in modulated songs arouse flies.
Do flies hear pulses with small amplitudes in modulated song? (**a**) To study this question, we generate a playback with all pulses set to the minimum amplitude of playbacks. If pulses at minimum amplitude are not heard by flies, then flies would behave similarly in response to songs with pulses at minimum amplitude as in response to songs with no pulses (sine-song-only condition). (**b**) In contrast, flies chain significantly more in response to songs with pulses at minimum amplitude than to songs with no pulses, demonstrating that flies respond to low-amplitude pulses in our playbacks. We show songs with *D. melanogaster* SAS as a control to illustrate the amplitude level of minimum pulses in playbacks. Note that the amplitude level of minimum pulses is consistent throughout playbacks, suggesting that differences in flies' responses to modulated SAS cannot be explained by perceptual omission of small-amplitude pulses. The white background marks the region of baseline chaining levels during silence, and the grey background marks the region of chaining in response to song playback. (**c**) The asterisks indicate significance levels: ** indicates $P < 0.005$, and *** indicates $P < 0.0005$. (In each case, we apply Bonferroni correction for multiple testing using a two-sided Wilcoxon signed-rank test.)

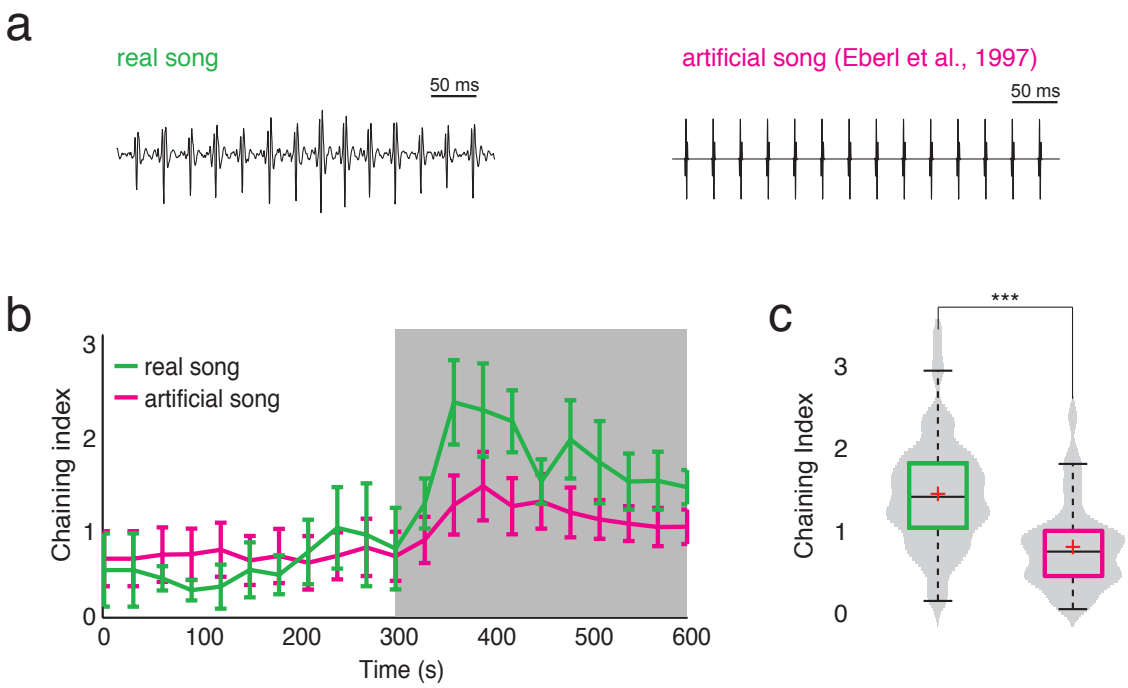



**Figure 4.10:** Real song excites *D. melanogaster* males more than artificial song.
(**a**) Illustration of song playbacks used in this experiment. We used a song recording of a *D. melanogaster* male and an artificial song published by.[99] (**b**) Chaining levels of *D. melanogaster* males during five minutes of silence (white background) followed by five minutes of song playback. (**c**) *D. melanogaster* males chain significantly more in response to real song than in response to artificial song ($P < 0.0001$, using a two-sided Wilcoxon signed-rank test).

# Chapter 5

# *Drosophila* females respond differently to random song amplitude structure than males

## 5.1 Introduction

Male flies court females by extending and vibrating one wing to produce a courtship song with species-specific characteristics.[29] Song characteristics influence behavioural responses to song.[29,99]

In fact, female *D. melanogaster* copulate more in response to song with their species IPI, than to songs with shorter or longer IPI.[29] This is true for *Melanogaster* males also, who chain more in response to song with their species IPI.[66] Flies have species-specific auditory responses to other song characteristics also, including frequency,[66] CPP[121] and KHC.[36]

We showed in Chapter 4 that females can make mating decisions based on SAS and males differentiate SAS in a similar way, which suggests that SAS is a communication signal in *D. melanogaster*.

However perceived amplitude can be subject to noise,[4] and it depends on the position of the sound source,[44] which implies that positional changes during courtship can affect perceived SAS and may introduce noise. We observe that positional changes are frequent during courtship: The male circles the female, sometimes singing in front of her, sometimes singing at her back. Researchers have shown that sounds that originate from females back are likely to be not perceived by the female ear.[44] This implies that some song may be occluded from female perception due to movement of

the male and the female. Additionally, distance between flies may affect perceived song amplitude, as perceived amplitude decays rapidly with distance.[125] Male flies may compensate for distance to a female by actively modulating their song amplitude with distance (AMD),[31] to sing louder when further away from a female.

Other animals, including birds, mammals, and amphibians,[3,5] compensate for distance by vocalising louder when farther away from receivers. Amplitude modulation with distance seems to be an evolutionary conserved strategy to account for distance between senders and receivers.

However even when counteracting distance with AMD, perceived SAS is still likely to be distorted because of several factors, including the rapid attenuation rate of perceived amplitude,[125] the position of the singer,[44] male–male competition[75] and environmental noise sources like wind.[4] We therefore examine whether flies can cope with noisy SAS.

## 5.2 Methods

### 5.2.1 Flies and experimental protocols.

We use the same methods for fly husbandry and the same experimental protocols for playback assays that we described in Section 4.2.1.

For copulation assays, we muted and deafened male flies by cutting their wings and arista.[29] Thus male flies in the copulation assay were unable to hear song playback, while females were able to hear song playback. We are thus concerned with female response to song for the copulation assay, as deafened males do not respond to song.[100] Before copulation experiments, we aged males in isolation for 3–7 days and aged females in groups for 3–7 days. We conducted copulation experiments throughout the day. We did not use blinding for analyzing playback assays.

We assess male flies auditory response to song using chaining. Chaining is a behavioural phenomenon in which a group of males, upon hearing song with species-specific characteristics start to court and chase one another.[66,99,100] (See Figure 5.2c for an illustration of the chaining assay.) We followed the chaining protocol described by Inagaki et al.[100] We collected male virgins within six hours after eclosion and muted them by cutting their wings above the anterior crossvein. Males aged in groups of eight animals of the same genotype for 3–5 days. We conducted chaining experiments

between 0 and 8 hours after light onset and determined the order of playbacks at the beginning of each session.

To avoid habituation, we tested each group of flies in only one playback. We controlled for mean playback amplitude by generating playback stimuli that have similar mean amplitude (±1%).

### 5.2.2 Materials

We describe our materials for song recording and for song playback in Section 4.2.2.

### 5.2.3 Data analysis

We automatically segmented recorded songs with FlySongSegmenter.[32] We analysed chaining with the tracker software ChaIN.[66]

### 5.2.4 Stimulus design

For testing whether SAS can be explained as uniform random variations, we randomised the SAS of a recorded *D. melanogaster* audio track with a MATLAB script that we make publicly available.[126] For randomising SAS, we proceed as follows

1. Detect pulses $P_\mathrm{i}$ in the audio track $S(t)$ with FlySongSegmenter.[32] The index $i$ gives the count of pulses in audio track $S(t)$.

2. We then determine amplitude maxima of pulses $A_\mathrm{i} = \max(P_\mathrm{i})$.

3. We then randomise the order of pulses by shuffling count $i$, so that we consider $A_r = \max(P_r)$, where $r = \texttt{rand(i)}$, and `rand` is the default random-number function in MATLAB, which in our release (8.1.0.604, R2013a) is the Mersenne Twister with the seed set to 0.

4. We then normalise pulse amplitude. Pulse amplitude maxima and minima can have different absolute values, and we decided to set them to the same absolute value after normalisation. We normalise maxima and minima separately in two steps. For normalising maxima, we (1) determine pulse maxima, $A_\mathrm{i} = \max(P_\mathrm{i})$. We then (2) split the audio track $S(t)$ along the horizontal axis to generate split

audio tracks, which represent positive or negative amplitude portions, to normalise amplitude maxima and minima separately. We generate split audio track $S_{\mathrm{p}}(t)$ which consists of all nonnegative $S(t)$. (3) We normalise pulses in split audio track $S_{\mathrm{p}}$ by dividing them by their amplitude maximum $A_{\mathrm{i}}$. For (4) normalising pulse amplitude minima, we determine the pulse minima $\min(P_{\mathrm{i}})$. We then (5) split the audio track $S(t)$ along the horizontal axis to generate $S_{\mathrm{n}}$, which consists of all negative $S(t)$ and (6) normalise pulses in the split audio track $S_{\mathrm{n}}$ by dividing them with their amplitude minimum $\min(P_{\mathrm{i}})$. Finally, we (7) join the normalized split audio tracks $S_{\mathrm{p}}$ and $S_{\mathrm{n}}$ to generate constant-amplitude audio track $S_{\mathrm{c}}(t)$, such that $S_{\mathrm{c}}(t) = S_{\mathrm{p}}$ for all $t$ that satisfy $S(t) \geq 0$ and $S_{\mathrm{c}}(t) = S_{\mathrm{n}}$ for all $t$ that satisfy $S(t) < 0$.

5. In our final step, we apply the randomised order of pulse amplitude maxima $A_{\mathrm{r}}$ on the piecewise-constant amplitude audio track $S_{\mathrm{c}}(t)$ to generate random amplitude audio track $S_{\mathrm{r}}(t) = S_{\mathrm{c}} \times A_{\mathrm{r}}$.

We designed playback stimuli from recordings of courtship song of *D. melanogaster* (see Figure 5.1a; Note that Figure 5.1 is a repetition of Figure 3.3.). The song statistics of the song recording are in the normal range of *D. melanogaster* song with a mean pulse frequency of 192.3 Hz, a mean sine song frequency of 166.1 Hz, a mean inter-pulse interval of 35.8 ms and a mean number of cycles per pulse of 1.4 (compare e.g.[32]). We controlled the mean amplitude of playback stimuli by computing the mean amplitude of each playback stimuli and subsequently adjusting the mean amplitudes to match each another.

## 5.3 Results

### 5.3.1 Song amplitude structure is not random

To answer our question on whether randomising the temporal sequence of pulse amplitudes affects the attractiveness of song, we randomise the temporal sequence of pulse amplitudes in a recording of *D. melanogaster* song. (See Section 5.2.4 for details on randomising the temporal sequence of pulse amplitudes.) We show a sample song trace with real song amplitude structure (SAS) and randomised SAS in Figure 5.1a. We introduce noise to SAS by randomising the temporal structure of SAS. Importantly, modulation of the temporal sequence of pulse amplitudes does not change

other song parameters, nor does it change the overall amplitude of song (see Figure 4.8). However the temporal sequences of songs with real and randomised SAS differ significantly (see Figure 5.1b), which demonstrates that the temporal sequence of song amplitude in real song is not random.

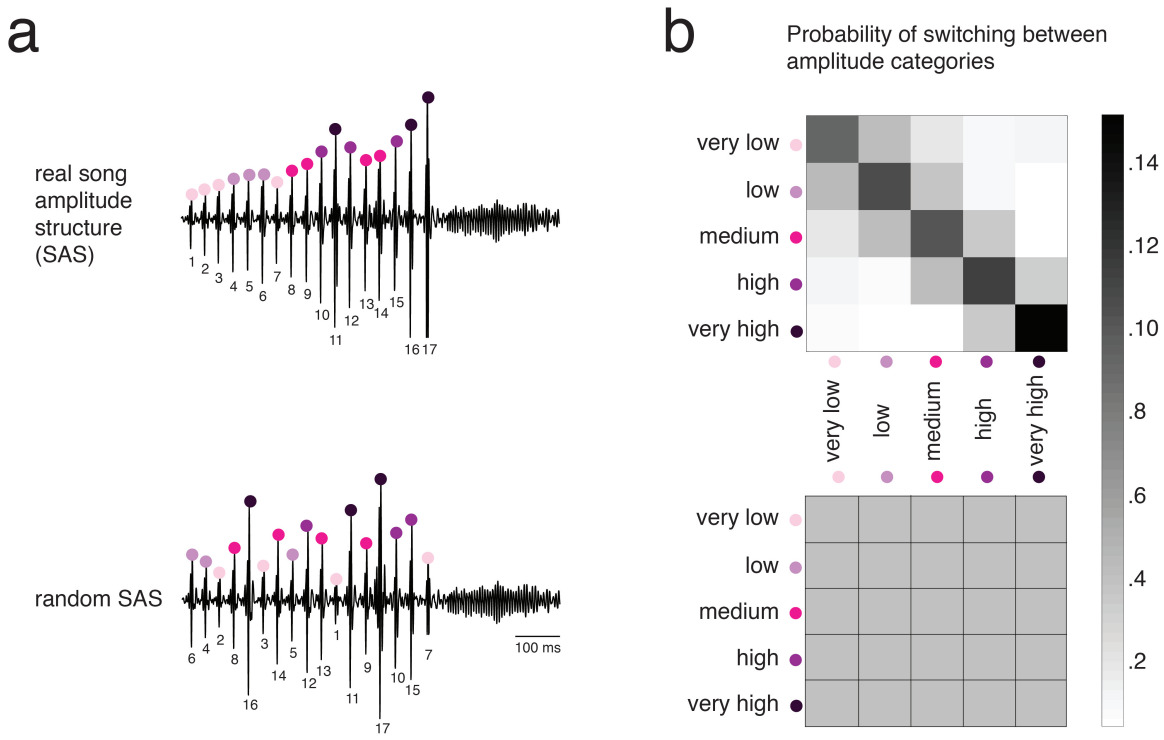



**Figure 5.1:** Song amplitude structure is not random.
**(a)** Recorded courtship song of a *melanogaster* male. Dots show the maximum amplitude of pulses. The colour of circles represents amplitude categories of very low (light pink) to very high amplitude (purple). The same recording with randomised SAS (see Section 5.2.4). The numbers below each pulse illustrate randomisation. **(b)** Heat maps reveal the probability of switching between amplitude categories. The probability of switching is approximately uniform across amplitude categories for random SAS. The switch probabilities of real SAS differ significantly from random SAS ($P < 0.0001$, Chi-Square Goodness-of-Fit Test). Figure 5.1 is a repetition of Figure 3.3 .

### 5.3.2 Females and males differ in their response to randomised song amplitude structure

We investigate whether flies behavioural response is robust to noisy SAS. To assess flies behavioural responses we play back courtship song via a speaker to flies. See Figure 5.2a for an illustration of our song playback setup. We use standard behavioural assays to assess auditory responses of female and male flies.[4,29,35,66,99,100] In the copulation assay we play back song to male–female couples. Note that we mute males, who usually sing with a vibrating wing, by clipping their wings, and we thereby ensure

that flies hear only our playback. Additionally, we deafen males by removing their arista, which renders males unresponsive to song playback.[100] As males cannot hear, song playback can affect the female mating decision only, as females in our copulation assay have intact ears. (See Figure 5.2b for an illustration of our copulation assay.) We assess male responses to song playback with the chaining assay.[100] (See Figure 5.2c for an illustration of the chaining assay.)

We now examine whether randomisation of SAS affects female mating choice, and find that it does not (Figure 5.1d). However female mating choice is affected by SAS (see Chapter 4), which suggests that females process SAS. We then examine whether randomisation of SAS affects male chaining response, and expect that—similar to females—males do not respond differently to playbacks with randomised SAS and real SAS. However to our surprise, males chain significantly less in response to randomised SAS ($P < 0.001$, two-sided Wilcoxon rank sum test, see Figure 5.1e), which suggests that randomisation of SAS affects males' but not females' behavioural response to song.

### 5.3.3 Sex differences in response to randomised song amplitude structure are robust

The sex difference we find in the response to playbacks with real and randomised SAS could be due to the specifics of our randomisation: we randomised the temporal sequence of SAS and thus produced a new temporal sequence. To test whether the temporal sequence of our first randomisation can explain our results we generated a second playback with randomised SAS that had a different sequence than our first playback with randomised SAS. We again find that the fraction of copulating pairs is similar across playback conditions, which suggests that females do not behaviourally discriminate playbacks with real SAS and playbacks with randomised SAS (see Figure 5.3a). We again find that males chain more in response to playback with real SAS than to playback with randomised SAS ($P < 0.001$, WR, see Figure 5.3b). This suggests that the sex difference in behavioural responses to randomised SAS cannot be explained by the specific sequence of randomisation, but may be due to randomisation itself.

However the sex difference may be explained by the specific song we use for playback experiments. To test whether this is true and our results could be explained by our choice of playback, we choose a different playback and generate a version of this playback with randomised SAS. We again find that the fraction of copulating

pairs is similar across playbacks, which suggests that females do not behaviourally discriminate between playbacks (see Figure 5.4a). Males chain more in response to the playback with real SAS than to the playback with randomised SAS ($P < 0.001$, WR, see Figure 5.4b), which suggests that our choice of playback cannot explain the sex difference in response to playbacks.

Both replications suggest that the sex difference in behavioural responses to playbacks with randomised SAS is robust.

## 5.4 Discussion

Male fruit flies behaviourally discriminate song with real SAS from song with randomised SAS, whereas females do not, suggesting that processing of noise in SAS is sexually dimorphic. Why should processing of noise in SAS be sexually dimorphic? Males respond to song by chasing and courting one another, which is a behaviour that has no clear ethological benefit, as courtship is energetically costly and same-sex courtship does not promise reproductive success. Therefore it may be beneficial for males to be selective when responding to song. Indeed, male *D. melanogaster* exhibit behavioural selectivity to song when they chain more in response to song with their species IPI than to song with different IPI.[66,72] To reduce mating errors, male *D. melanogaster* may have evolved to respond more selectively to noisy SAS than females. Chaining involves male movement and positional changes, which are known to affect song amplitude[31] and perceived amplitude.[44] Thus male sensitivity to noisy SAS may enable *D. melanogaster* males to stop courting other males when SAS is distorted by fly movement. But it remains to be tested whether SAS is indeed distorted by movement and we discuss potential methods to test this in Section 7.1.

To our knowledge we are the first to claim that behavioural responses to song can differ between sexes. Our claim is limited by the fact that we use different behavioural paradigms to asses female responses to song (copulation) and male responses to song (chaining). Thus differences in responses could be explained by differences in the experimental paradigm. However we find that female and male responses in the two paradigms are comparable when we test how females and males respond to SAS and IPI (see Chapter 4): both females and males respond most to their species song characteristics. This suggests that the two paradigms can produce comparable results, and the difference in experimental paradigm may not explain the difference we observe in female and male responses to randomised SAS. Note that auditory neurons can be

sexually dimorphic[69,71,72] which suggests that there could be an anatomical and physiological basis for differences in behavioural responses to song. It will be interesting to investigate a potential difference in the auditory processing of randomised SAS in female and male auditory neurons, which are known to be sexually dimorphic.[71,72]

Figure 5.2d seems in direct conflict with Figures 4.5a and 4.6. Figure 5.2d demonstrates that *D. melanogaster* copulate at similar levels when exposed to recorded SAS and randomised SAS, and Figures 4.5a and 4.6 show that *D. melanogaster* copulate at different levels when hearing songs with different SAS modulations. The fact that females respond robustly to random SAS suggests that they can compensate for noise in SAS, and the result that females respond distinctly to songs with different SAS modulations, implies that females can process amplitude modulations. The SAS modulations that we apply in Chapter 4 are regular structures, whereas randomisation of SAS removes structure. It could be that random and structured amplitude modulations are processed in distinct auditory pathways in females. If random amplitude modulations (as in Figure 5.2d) were processed in a different pathway than structured modulations (as in Figure 4.5a), behavioural responses to the two types of modulations could be distinct, which might explain our observations. Note that *D. melanogaster* possess multiple pathways that process distinct auditory characteristics of song: Researchers find that first order auditory neurons (JO) respond specifically to song frequencies[15] and third order auditory neurons respond specifically to IPI.[70,72] This demonstrates that flies have multiple auditory pathways that can process different song characteristics.

An alternative explanation for our findings could be that recorded song is noisy[31] and does not differ significantly from randomised SAS.

However we demonstrate that males respond less to song with randomised SAS than to song with real SAS, which suggests that males are sensitive to randomisation in SAS. Note that our playbacks with real SAS may also contain noise as we do not control for the distance between microphone and singing male, and this may introduce noise to the recorded song amplitude.[31] Yet we find that males chain robustly in response to our recordings, which suggests that they may be able to tolerate some noise in SAS. Therefore we further investigate male responses to noise in SAS in Chapter 6.

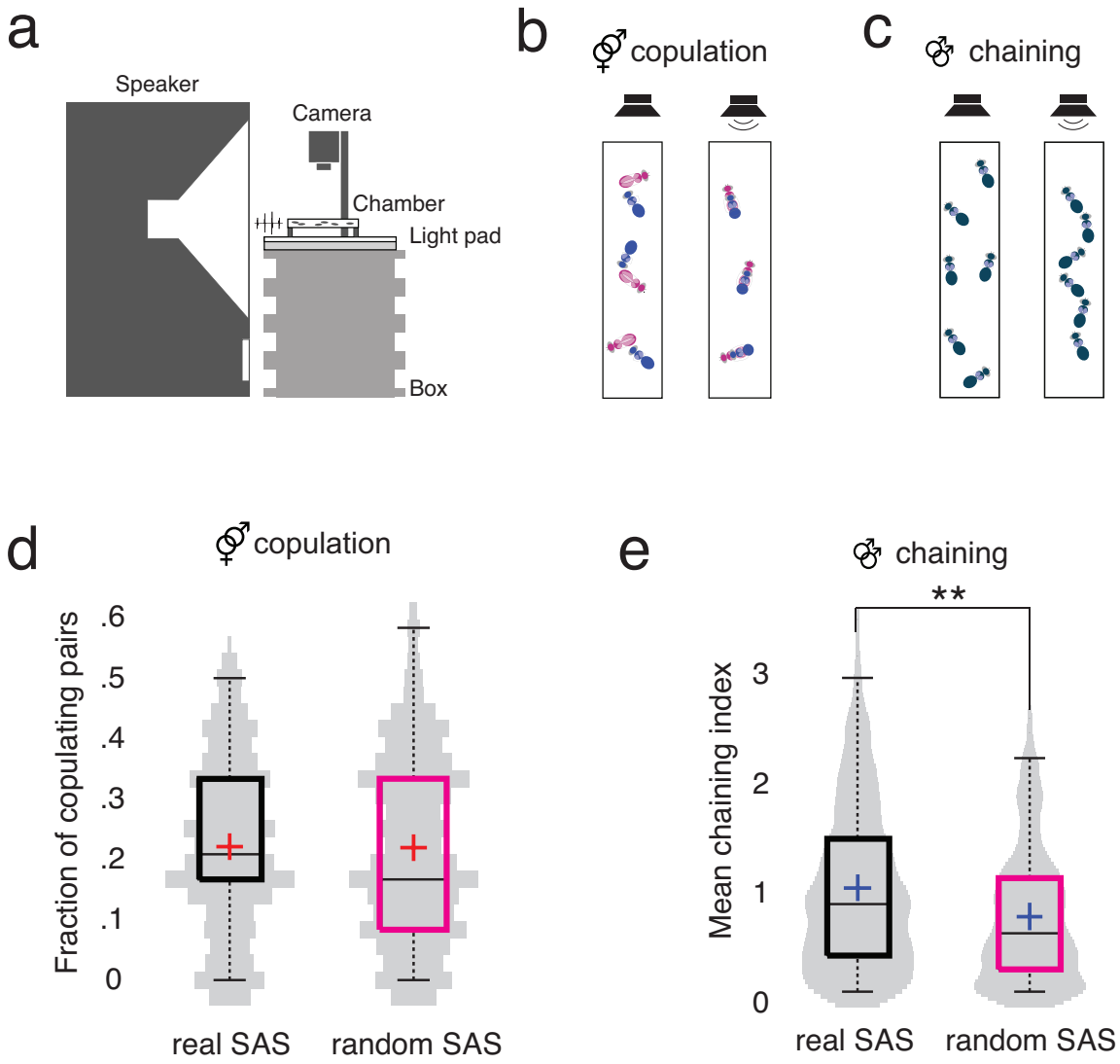



**Figure 5.2:** Female flies respond differently to random song amplitude structure than male flies. **(a)** Schematic of playback setup. **(b)** Schematic of copulation assay to test female response to song playback. Males in the copulation assay are muted by removing the wings and deafened by removing arista. Males without arista do not respond to playback,[100] which is why this assay assesses only female response to playback. **(c)** Schematic of chaining assay for testing auditory responses in *D. melanogaster* males.[99,100] **(d)** Fraction of copulating pairs in response to song playback. Female mating decision is not affected by randomisation of SAS (a p-value of $P \approx 0.936$, $F \approx 0.01$ in a one-way anova, and $n_c = 120$, where $n_c$ is the number of couples with one male and one female). Mating frequency is significantly larger during playback of both real and random SAS than during silence control [$P < 0.0001$, two-sided Wilcoxon rank sum test (WR)]. **(e)** Male chaining[99] in response to song with real SAS and song with randomised SAS. In the chaining assay, six males are subjected to five minutes of silence followed by five minutes of song playback. We show the mean chaining index over the five minutes period of playback. A mean chaining index of 2 means that on average two males chain during playback. Note that chains often break which results in a chaining index of 0, which is why we obtain mean chaining indices with values near 1. Males increase chaining in response to playback ($P < 0.0001$, WR). Male flies chain significantly more in response to song with real SAS ($n_g = 19$, where $n_g$ is the number of groups with six males) than to song with randomised SAS ($n_g = 16$; $P < 0.01$, WR). We show the distribution of chaining data as grey kernel density plots, which are mirrored across the vertical axis and as boxplots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 $\times$ IQR. The asterisks indicate level of significance: ** indicates a p-value of $P < 0.01$ (WR).

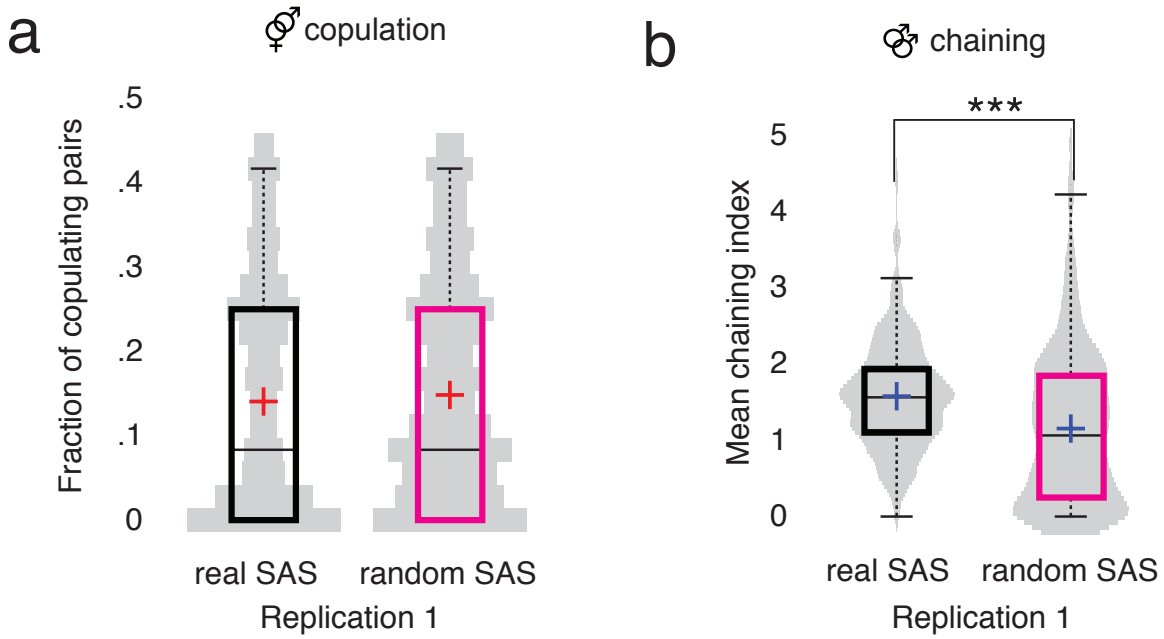



**Figure 5.3:** Sex differences in behavioural responses to randomised SAS are robust for a different randomisation sequence.

(**a**) The fraction of copulating pairs is similar in response to song with real SAS and in response to song with randomised SAS (a p-value of $P \approx 0.721$, $F \approx 0.13$ in a one-way anova, and $n_c = 108$, where $n_c$ is the number of couples with one male and one female). This replicates the results we show in Figure 5.2d. For the condition 'real SAS' in Replication 1 we played back the same song as in the experiment we present in Figure 5.2. For the condition 'random SAS' we played back a song with a different randomisation sequence than for the experiment we present in Figure 5.2. (**b**) Male chaining[99] in response to song with real SAS and song with randomised SAS. We use the same playbacks as in (**a**). In the chaining assay, six males are subjected to five minutes of silence followed by five minutes of song playback. We show the mean chaining index over the five minutes period of playback. Males increase chaining in response to playback ($P < 0.0001$, WR). Male flies chain significantly more in response to song with real SAS ($n_g = 28$, where $n_g$ is the number of groups with six males) than to song with randomised SAS ($n_g = 24$; $P < 0.001$, WR). We show the distribution of chaining data as grey kernel density plots, which we mirror across the vertical axis and as boxplots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 × IQR. We show the distribution of the fraction of copulating pairs in a similar way. The asterisks indicate level of significance: *** indicates a p-value of $P < 0.001$ (WR).

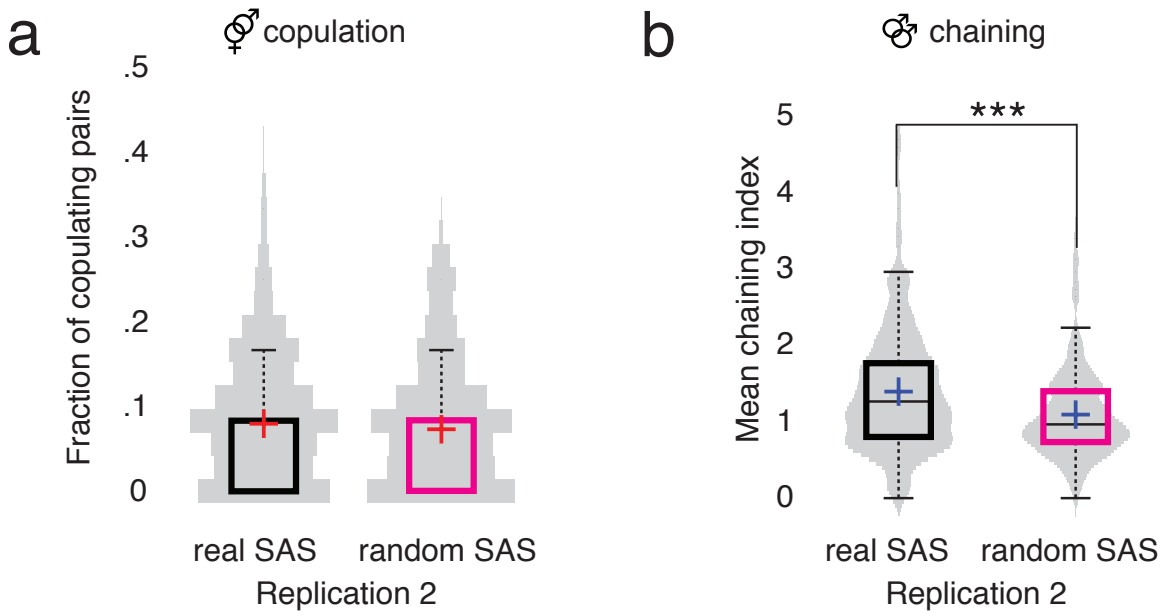



**Figure 5.4:** Sex differences in behavioural responses to randomised SAS are robust for a different song.

(**a**) The fraction of copulating pairs is similar in response to song with real SAS and in response to song with randomised SAS (a p-value of $P \approx 0.468$, $F \approx 0.51$ in a one-way anova, and $n_c = 168$). For the condition 'real SAS' in Replication 2 we played back a recording from a different *D. melanogaster* male than for the experiment we show in Figure 5.2. For the condition 'random SAS' we randomise SAS. (**b**) Male chaining[99] in response to song with real SAS and song with randomised SAS. We use the same playbacks as in (**a**). In the chaining assay, six males are subjected to five minutes of silence followed by five minutes of song playback. We show the mean chaining index over the five minutes period of playback. Males increase chaining in response to playback ($P < 0.0001$, WR). Male flies chain significantly more in response to song with real SAS ($n_g = 24$) than to song with randomised SAS ($n_g = 20$; $P < 0.001$, WR). We show the distribution of chaining data as grey kernel density plots, which we mirror across the vertical axis and as boxplots. A red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates $1.5 \times$ IQR. We show the distribution of the fraction of copulating pairs in a similar way. The asterisks indicate level of significance: *** indicates a p-value of $P < 0.001$ (WR).

# Chapter 6

# Male *Drosophila* can restore random song amplitude structure

## 6.1 Introduction

Male flies vibrate their wings to produce a courtship song with species-specific characteristics.[25] Earlier in this thesis, we showed that SAS is a communication signal in *Drosophila* that can signal muscle power (see Chapter 3) and species type (see Chapter 4). Noise may affect perceived amplitude[4,31,44,75] and we mimicked noise by randomising SAS in Chapter 5. We found that randomisation of SAS can affect behavioural responses to song playbacks. Specifically randomisation of SAS in playbacks affects the behavioural response of males. Males can respond to song by forming courtship chains[99] and we show in Chapter 5 that males chain more in response to playbacks with real SAS than to playbacks with randomised SAS. This suggests that playbacks with randomised SAS can impair male chaining responses, which may be because the communication signal in real SAS is disrupted by randomisation.

Disrupted acoustic signals can be restored by animals in a perceptual process known as '*auditory perceptual restoration*'.[102] The ability to restore disrupted auditory information can help animals to communicate in noisy environments.[5,76,102] Auditory perceptual restoration has been demonstrated in humans,[77,102] non-human primates,[78,79] cats,[80] rodents[81] and birds,[82,83] which suggests evolutionary conversation of the ability to auditory restore missing information.[76] Despite evidence for evolutionary conversation of auditory restoration, evidence for auditory restoration in insects has not yet been presented (to our knowledge). Our present work suggests that *Drosophila melanogaster* males can restore auditory information missing from

SAS. Male *Drosophila* may use a process related to auditory restoration for restoring auditory information missing from SAS.

## 6.2 Methods

### 6.2.1 Flies and experimental protocols

We use the same methods for fly husbandry and the same experimental protocols for playback assays as we describe in Section 4.2.1.

### 6.2.2 Materials

We describe our materials for song recording and for song playback in Section 4.2.2.

### 6.2.3 Data analysis

We compute the mean information[127] that the amplitude category $A_x$ of a pulse conveys about the amplitude category $A_y$ of a subsequent pulse as mutual information

$$I(A_x, A_y) = \Big( \sum_{A_x, A_y} p(A_x, A_y) \times \ln \frac{p(A_x, A_y)}{p(A_x)p(A_y)} \Big) \times \frac{1}{n_c^2} \,, \tag{6.1}$$

where $p(A_x, A_y)$ is the probability of a pulse in amplitude category $A_y$ following on a pulse in amplitude category $A_x$, the quantity $p(A_x)$ is the probability of a pulse in amplitude category $A_x$, the quantity $p(A_y)$ is the probability of a pulse in amplitude category $A_y$, and $n_c$ is the number of amplitude categories (where $n_c = 5$ in our example). We square $n_c$ to give us the number of possible transitions between amplitude categories, and we include transitions in the same category. We bin data such that each amplitude category had approximately the same number of constituents ($\pm 5\%$). For the song data we analysed this constitutes the following amplitude categories $A_i$ for peak amplitudes $P_A \in [0, 1]$: (1) $A_1 = [0, 0.0365]$, (2) $A_2 = (0.0365, 0.127]$, (3) $A_3 = (0.127, 0.228]$, (4) $A_4 = (0.228, 0.396]$ and (5) $A_5 = (0.396, 1]$.

We automatically segmented recorded songs with FlySongSegmenter.[32] We analysed chaining with the tracker software ChaIN.[66]

### 6.2.4 Stimulus design

For generating playback with alternating pulse trains with real SAS and randomised SAS, we proceed as follows:

1. Detect pulses $P_\mathrm{i}$ in the audio track $S(t)$ with FlySongSegmenter.[32] The index $i$ gives the count of pulses in audio track $S(t)$.

2. Specify the fraction $F_R = \frac{n_\mathrm{Rand}}{n_P}$ of pulses with randomised SAS, where $n_\mathrm{Rand}$ is the number of pulses with randomised SAS and $n_P$ is the total number of pulses in the playback.

3. Specify the number $n_\mathrm{rand}$ of pulses in a pulse train with randomised SAS.

4. Compute the number $n_\mathrm{real}$ of pulses in a pulse train with real SAS as function of $n_P$, $F_R$ and $n_\mathrm{rand}$, such that $n_\mathrm{real} = \frac{n_P \times (1-F_R) \times n_\mathrm{rand}}{n_P \times F_R}$.

5. Specify whether we want the playback to start with a pulse train with real SAS or with a pulse train with randomised SAS.

6. Identify the indices of pulses $I_\mathrm{Rand}$ that are to be randomised. For example we have a playback with a total number of 40 pulses ($n_P = 40$). We specify the number of pulses with randomised SAS $n_\mathrm{rand}$ to be 10 and the fraction of pulses with randomised SAS $F_R$ to 0.5. We then compute the number of pulses in a pulse train with real SAS to be $n_\mathrm{real} = 10$. We specify that the playback should start with a pulse train with randomised SAS. We can then determine the indices $I_\mathrm{Rand}$ as $I_\mathrm{Rand} = [1...10, 21...30]$ in MATLAB notation.

7. Now randomise the amplitudes of pulses $P_\mathrm{i}$ with indices $i \in I_\mathrm{Rand}$. For randomising amplitudes we use the same algorithm as described in Section 5.2.4.

Our MATLAB code for generating playback stimuli with alternating random and real SAS is publicly available.[128]

## 6.3 Results

### 6.3.1 Song amplitude structure loses information when randomised

Animals can restore acoustic signals that are missing information.[102,129] Therefore we investigate whether there is information missing from randomised SAS which is present in real SAS.

To test whether randomised SAS misses information stored in real SAS, we compute the information of both real and randomised SAS. For details on computing the information of SAS see Section 6.2.3. We find that randomisation of SAS removes information stored in real SAS. In real SAS one can approximately predict what amplitude a pulse will have given the amplitude of the preceding pulse. Thus in real SAS each pulse stores information about the amplitude of the next pulse. We can quantify this information in bits, and each pulse in real SAS stores a mean of 0.53 bits of information about the amplitude of the next pulse. In contrast, pulses in random SAS store no information (0 bit) about the amplitude of the next pulse. Thus information is missing from randomised SAS, and this makes random SAS an appropriate stimuli for testing whether flies can restore missing auditory information. Therefore, we investigate whether male flies can restore information missing in random SAS. Importantly, missing auditory information can only be restored if it is sufficiently brief.[79,81,82,102,130] It has been reported that animals lose their ability to restore missing information from auditory signals if the missing information is longer than about 500 milliseconds.[102,131]

### 6.3.2 Auditory responses are robust to brief amplitude distortions

We investigate whether *Drosophila* males chaining response is affected by the duration of pulse song with random SAS by presenting random SAS either in brief pulse trains of about 350 ms duration (comprising 10 pulses) or in long pulse trains (about 2.5 minutes or longer). We expect that males respond similarly as we observed in Chapter 5 and chain less when hearing song with random SAS, than when hearing song with real SAS. Importantly, we present males with playbacks that contain both pulse song with real and random SAS and we illustrate which parts of playbacks are randomised in Figure 6.1a. To test whether male flies respond differently when pulses with random SAS follow on pulses with real SAS than when pulses with real SAS

follow on pulses with random SAS we create two control stimuli that either start with a long pulse train with random SAS or end with a long pulse train with random SAS. Stimuli with short pulse trains with random SAS (about 350 ms) alternate between random SAS and real SAS (about 100 ms to 350 ms).

The ratio $r_{\frac{\text{rand}}{\text{real}}}$ of the number of pulses with random SAS to the number of pulses with real SAS is the same for all stimuli. Thus if duration of pulse trains with random SAS did not affect auditory responses of *Drosophila* males, we would expect that the behavioural response to playback with the same ratio $r_{\frac{\text{rand}}{\text{real}}}$ would be the same. In contrast, we observe that *Drosophila* males chain more in response to playback with short pulse trains with random SAS than in response to playbacks with long pulse trains with random SAS (see Figure 6.1b–d), which suggests that *Drosophila* can restore missing information in SAS if the information is missing for a brief period of time only.

The difference between short and long pulse trains only becomes significant when the playback consists of more random than real SAS (more than 50% random SAS, see Figure 6.1b–d). This may be because we compare chaining responses to stimuli that consist of both real and random SAS. We design control stimuli such that a long pulse train with random SAS follows a long pulse train with real SAS or vice versa (see Figure 6.1a) and expect that male flies chain at lower levels during random SAS than during real SAS (compare Figure 5.2e). We thus expect chaining responses to be between chaining responses to real and random SAS. Consequently, the higher the amount of real SAS in control stimuli, the more similar chaining will be to real SAS chaining levels. Thus the more real SAS is present in a playback stimulus, the more similar we expect chaining levels to be to real SAS chaining levels. This is why we may require the percentage of random SAS to exceed 50% to observe a significant difference between control stimuli and restoration candidates (Figure 6.1c–d).

During chaining, males chase and court one another[100] and are in a state of sexual excitement[72] which can be self-perpetuating.[132] Therefore it is reasonable to ask whether self-perpetuating sexual excitement can outlast short pulse trains with random SAS. We thus investigate whether flies remain sexually excited within the sound of silence. If self-perpetuating sexual excitement caused increased chaining levels in playbacks with short pulse trains with missing auditory information, then we would expect similar chaining levels when we replace pulse trains with random SAS with periods of silence. In contrast, we see a significant reduction in chaining response when we replace pulse trains with random SAS with periods of silence (Figure 6.1d),

which demonstrates that self-perpetuating sexual excitement cannot explain the increased chaining levels we see in playback with short trains of pulses with random SAS. Note that we do not expect auditory restoration when the signal is interrupted by silence gaps, as other animals do not restore auditory information when signals are interrupted by silence gaps.[79,81,82] In agreement with this, we find that *Drosophila* males do not restore signals that are interrupted by silence gaps.

### 6.3.3 Auditory responses depend on the durations of both distorted and intact SAS

We asked next whether the duration of pulse trains with random SAS had an effect on behavioural responses of male *D. melanogaster*. See Figure 6.2a for an illustration of our playback stimuli. In our initial experiments (Figure 6.1) we chose pulse trains with random SAS to comprise 10 pulses, resulting in pulse train durations of about 350 ms duration, which is a duration of missing auditory information that can be restored by vertebrates.[79,81,82,130] Note that vertebrates restore missing auditory information that is shorter than about 500 ms.[79,81,82,130] Therefore we expect that flies lose the ability to restore information missing from pulse trains with random SAS when we increase the duration of pulse trains with random SAS. Indeed, we see significantly decreased chaining in response to pulse trains with random SAS comprising 40 pulses (approximately 1400 ms, $P < 0.0001$, two-sided WR) compared to pulse trains with random SAS comprising 10 pulses (approximately 350 ms) as we show in Figure 6.2b. Importantly, we keep the overall amount of pulses with random SAS constant throughout playback conditions, with each playback containing 75% pulses with random SAS. Therefore the difference in chaining is not due to a difference in the amount of random SAS, as this is kept constant, but instead the difference in chaining is due to the different duration of pulse trains with random SAS. Interestingly, male flies chain at similar levels when presented with pulse trains with random SAS comprising 10 pulses (approximately 350 ms) or 20 pulses (approximately 700 ms, $P \approx 0.69$, two-sided WR), which suggests that fruit flies can restore missing information from SAS over at least 700 ms, but less than 1400 ms. In addition, we find that *Drosophila* males also chain significantly less in response to pulse trains with random SAS comprising 5 pulses (approximately 175 ms, $P < 0.0001$, two-sided WR) compared to chaining to playbacks with pulse trains with random SAS comprising 10 pulses. This seemingly surprising result can be explained by the fact that the surrounding information from pulses with real SAS is scarce, with only two pulses with real SAS surrounding 5 pulses with random SAS. Auditory restoration requires

sufficient context from a surrounding signal,[79,102] which likely is not sufficient with two pulses with real SAS. Thus reduced chaining in response to playbacks with pulse trains of random SAS comprising 5 pulses is likely due to the insufficient amount of surrounding signal of real SAS.

## 6.4 Male flies do not restore song from white noise

Mammals and birds can restore auditory information from white noise that contains frequencies present in the signal.[77–83] However white noise contains a wide spectrum of frequencies, thus to restore auditory information from white noise mammals and birds need to actively filter and amplify relevant frequencies that fill in the noise gap.[102] Thus we asked whether *Drosophila* males can restore auditory information from white noise. To test this we generated playback stimuli similar to those presented in Figures 6.1 and 6.2, but we replaced the pulse trains with random SAS with white noise (see Figure 6.3a). If fruit flies were able to restore auditory information from white noise, we would expect them to show increased levels of chaining in response to playback with short periods of white noise.[102] In contrast however, we see that flies do not increase chaining in response to playback with short periods of white noise, but instead show markedly reduced chaining levels when compared to chaining in response to real song and when compared to control song that starts as real song and switches to white noise after 25% of pulses (Figure 6.3b–c). In fact, replacing all pulses with white noise, while keeping sine elements in the playback, renders playback completely unattractive with males not responding to playback ($P \approx 0.85$, WR), which is in agreement with males not chaining in response to sine song[66] (see also Figure 4.9). Our findings demonstrate that fruit flies—unlike vertebrates—can not restore auditory signals from white noise.

## 6.5 Discussion

Fruit flies can process courtship song even when loud noise occludes song,[4] which suggests fruit flies have evolved auditory mechanisms for robust processing of relevant auditory stimuli. Specifically, fruit flies seem capable of auditory streaming.[4] That is, fruit flies can separate signal (courtship song) from noise,[4] which is a process related

to auditory restoration.[102] Our work provides some evidence for auditory restoration in *Drosophila melanogaster* males when the restored auditory information closely matches real courtship song, differing only in SAS.

One obvious question to ask is whether the phenomena we describe are indeed processes related to auditory restoration. Mammals and birds can restore auditory information from white noise and we show that fruit fly males cannot restore auditory information from white noise (see Figure 6.3), which suggests that the phenomena we describe in fruit flies are different from auditory restoration that has been studied in vertebrates. This may be because the auditory system of insects is more specialised than that of vertebrates. The auditory systems of insects evolved to respond specifically to auditory signals that are relevant to them.[134] Courtship songs are such relevant signals.[134] Insects can extract relevant auditory information with a smaller number of neurons than vertebrates.[134] Auditory specialisation in insects is an efficient way to process relevant information, but this specialisation may come at the cost of not being able to process auditory stimuli, like white noise, that are not relevant to insects.[134] Thus the specialised auditory system of insects may impede auditory restoration from white noise in *Drosophila*.

We show that randomisation of SAS removes auditory information and that male flies chain less in response to playbacks with randomised SAS than in response to playbacks with real SAS (see Chapter 5), which suggests that randomisation of SAS can affect behavioural responses of male flies to playback. In this chapter we show that male responses to playback can be robust to randomisation of SAS if pulse trains with randomised SAS are short and alternate with pulse trains with real SAS. The conditions we find necessary for males to chain to song with randomised SAS are similar to those that are necessary for auditory restoration in mammals and birds,[102] which may suggest that restoration of SAS could be related to auditory restoration in other animals.

If restoration of SAS in male *Drosophila* were related to auditory restoration in other animals, we would expect to find additional similarities between the two phenomena. However, when mammals restore sounds that are missing auditory information their auditory neurons have activity patterns that are similar to when the animal hears sounds without missing auditory information.[131] Auditory neurons can respond to missing auditory information as if it were a continuous signal when they are restoring auditory information.[131] Thus auditory restoration can exhibit both neuronally[131] and behaviourally.[102] Our work gives some evidence for behavioural restoration of SAS in male *D. melanogaster*. It would be interesting to test whether

auditory neurons in *Drosophila* males can respond to random SAS as if it were real SAS, as this would give neuronal evidence for restoration of SAS in *Drosophila* males.

To test whether there is neural evidence for restoration of SAS in fly males, one would have to identify auditory neurons that can respond differently to playbacks with real and randomised SAS. Our result that females and males differ in their behavioural response to randomised SAS (see Chapter 5) may help to identify candidate auditory neurons, as our findings suggest that processing of randomised SAS may be sexually dimorphic. Therefore we expect sexually dimorphic auditory neurons to be involved in the processing of randomised SAS. Not all auditory neurons are sexually dimorphic, but sexual dimorphisms have been described for third-order and fourth-order neurons [1]. Primary and secondary auditory neurons do not show sexual dimorphisms on an anatomical level.[69] Thus candidate neurons for processing randomised SAS may be found on the third-order and fourth-order level of auditory processing in *Drosophila*.

[1]Neurons involved in auditory processing in *Drosophila* are described as 'third-order' and 'fourth-order'.[69,71,72] Researchers call JO auditory neurons 'primary' and they call AMMC neurons 'secondary' auditory neurons.[69] Primary auditory neurons are believed to connect to secondary auditory neurons, which in turn are believed to connect to third-order auditory neurons (vPN1[72]), which then are believed to connect to fourth-order auditory neurons (pC1[72]).

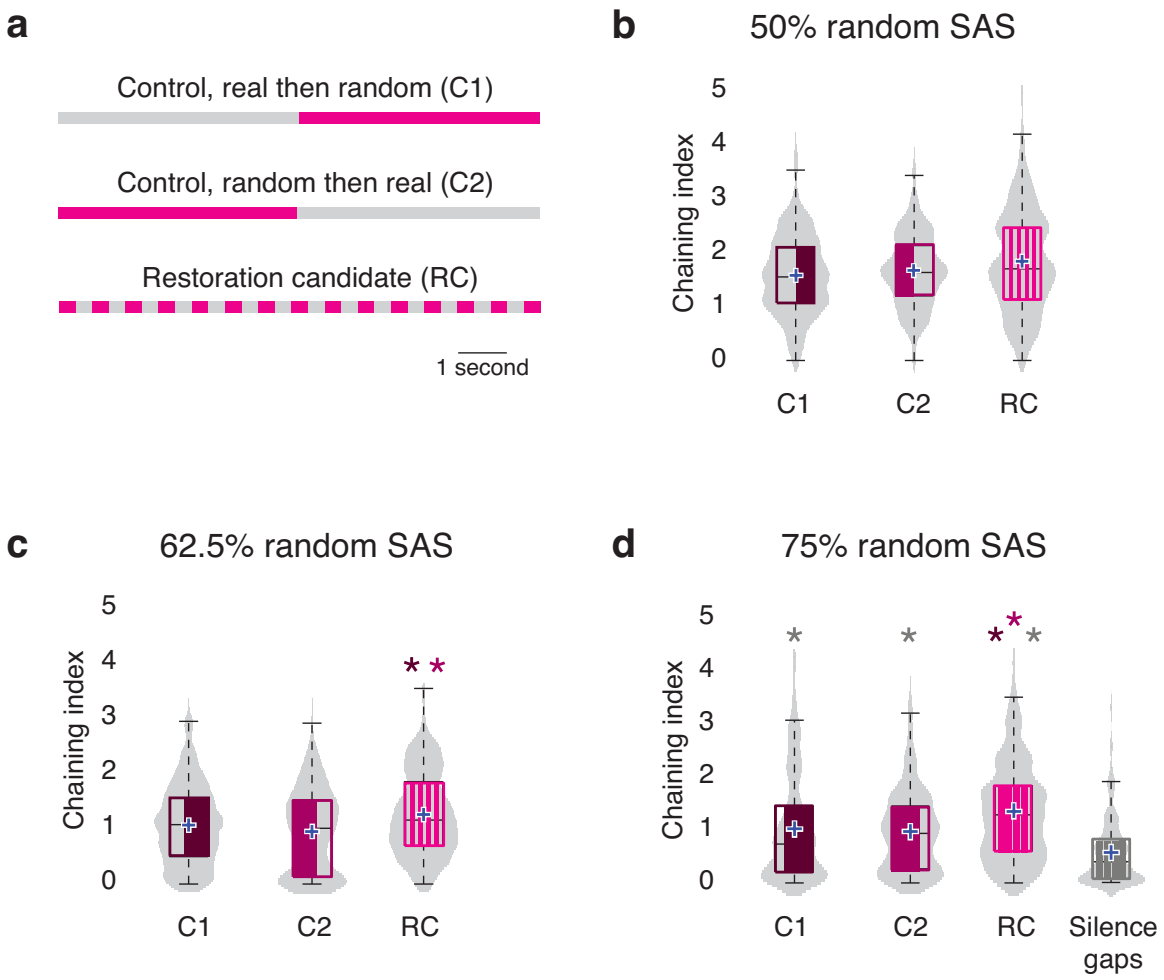



**Figure 6.1:** Fruit fly males can restore auditory information missing from SAS.
**(a)** Illustration of playback stimuli for testing auditory restoration of SAS. We take a recording of *D. melanogaster* courtship song and randomise the SAS of a fraction of the song (here 50% of the song's SAS is randomised). Bars illustrate which parts of the song's SAS are randomised: a grey bar indicates pulse song with real SAS and a pink bar indicates pulse song with random SAS. Note, that we only change the SAS of the playback, thus other elements of song as frequency, cycles per pulse and inter-pulse interval are unchanged and can not account for differences in auditory responses.[133] We use two controls for which we do not expect auditory restoration, and one stimuli that is a candidate for auditory restoration (see main text for explanation). The ratio of random SAS to real SAS is the same for playback stimuli. **(b)** Chaining responses towards song with 50% of pulses having random SAS. Flies show a non significant increase in chaining towards the restoration candidate ($P > 0.025$, $n_g = 42$, where $n_g$ is the number of groups of 6 males). **(c)** Chaining responses towards song with 62.5% of pulses having random SAS. Flies show a significant increase in chaining towards the restoration candidate ($P < 0.01$ and $n_g = 57$). **(d)** Chaining responses towards song with 75% of pulses having random SAS, respectively chaining in response towards song with 75% of pulses missing. Flies show a significant increase in chaining towards the restoration candidate with random SAS ($P < 0.003$, $n_g = 44$). Also flies chain significantly more to the restoration candidate with random SAS than to the restoration candidate with silence gaps ($P < 0.0003$, $n_g = 36$). We show the distribution of chaining data as grey kernel density plots, which are mirrored across the vertical axis and as boxplots, a white cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 × IQR. The asterisks indicate significant differences with a p-value of $P < 0.01$ (after correction for multiple testing using WR). The colour of the asterisk indicates which condition is significantly different. For avoiding redundancy we indicate significant difference between conditions at the larger of the two conditions only.

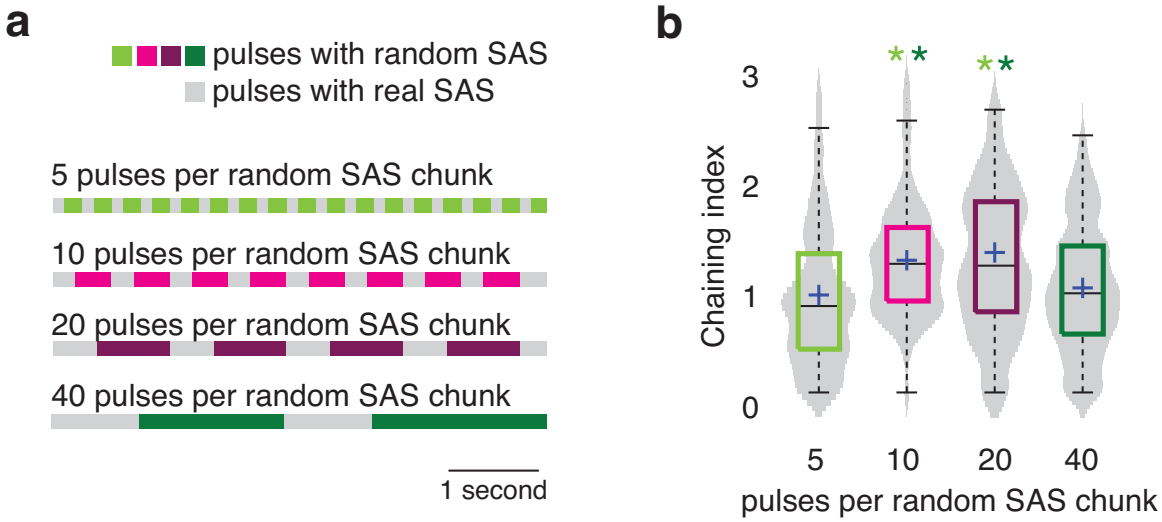



**Figure 6.2:** Duration of pulse trains with random SAS affects behavioural responses.
(**a**) Illustration of playback stimuli with varying number of pulses with randomised SAS, but constant ratio of real SAS to random SAS. Bars illustrate which parts of the song's SAS are randomised: a grey bar indicates pulse song with real SAS. Pink and green bars indicate pulse song with random SAS. Note, that both the duration of pulse trains with random SAS and the duration of pulse trains with real SAS varies between conditions. (**b**) Chaining responses to songs with varying duration of pulse trains with random and real SAS. Flies chain significantly more to song with 10 and 20 pulses per pulse train with random SAS than to other tested conditions ($P < 0.001$, two-sided Wilcoxon signed-rank test WR, $n_g = 71$, where $n_g$ is the number of male groups tested). Note that songs in this experiment contain 75% random SAS and 25% real SAS. We show the distribution of chaining data as grey kernel density plots, which are mirrored across the vertical axis and as boxplots, a red cross indicates the mean, a horizontal line indicates the median, a box indicates the IQR, and the whiskers indicates 1.5 × IQR. The asterisks indicate a significant difference ($P < 0.001$, two-sided WR) in chaining. The colour of the asterisk indicates which condition is significantly different. For redundancy we indicate significant difference between conditions at the larger of the two conditions only.

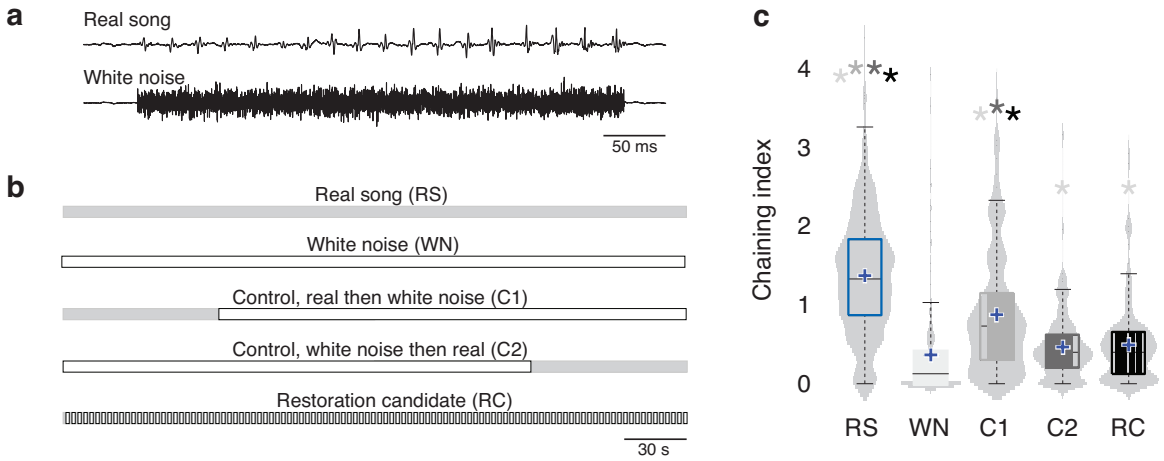



**Figure 6.3:** Male flies do not restore auditory information from white noise.
(**a**) Illustration of a train of pulses in real song and a version where the train of pulses is replaced by white noise. (**b**) Illustration of playback stimuli. Bars illustrate which parts of the song's pulses are replaced by white noise: a grey bar indicates pulse song with real SAS. White bars with black outline indicate white noise. We replace 75% of pulses in control stimuli and in the restoration candidate with white noise. (**c**) Chaining responses towards playback stimuli. Flies chain significantly more to the control that starts with real song than in response to the restoration candidate or the other control that starts with white noise. The asterisks indicate a significant difference ($P < 0.001$, two-sided Wilcoxon signed-rank test, $ng = 110$, where $n_g$ is the number of male groups tested) in chaining. The colour of the asterisk indicates which condition is significantly different. For avoiding redundancy we indicate significant difference between conditions at the larger of the two conditions only. The design of our graphs is similar to what we describe in Figure 6.1.

# Chapter 7

# Conclusions

We have learned that song amplitude structure (SAS) in *Drosophila* can signal muscle power (see Chapter 3) and species type (see Chapter 4) and that flies discriminate between their species SAS and other SAS, which demonstrates that SAS is a communication signal (see Chapter 4). Noise can interfere with signals[4,5] which is relevant for SAS as communication signal for two main reasons:

1. Movement can affect perceived song amplitude. Flies move during courtship and received song amplitude likely depends on the distance between flies[31] and their position to each other.[44] Our method of recording song with a stationary microphone does not capture the potential modulation of amplitude during movement. Therefore we discuss possible methods to investigate the question 'How does fly movement affect perceived song amplitude?' in Section 7.1.

2. Small amplitude pulses may not be perceived by flies. Fly ears respond to particle velocity (see in Section 1.7) and particle velocity decays rapidly with distance from the signalling source.[44] Therefore pulses with low particle velocity may be missed by flies. We ensured that pulses with low particle velocity are perceived by flies in our playback experiments (see Figure 4.9), but flies in the wild may miss small amplitude pulses, which may affect their responses to SAS. How much information can be missing from SAS for flies to still respond to SAS? We showed in Chapters 5 and 6 that flies can respond to SAS even when a considerable amount of information is missing, which suggests that flies are resilient to noise in SAS.

In Chapter 4 we showed that flies behaviourally discriminate SASs, which raises the question 'How do flies auditory process SAS?' which we discuss in Section 7.2. In Chapter 4 we also showed that the closely related species *D. melanogaster* and *D. simulans* behaviourally discriminate each others species SAS, which suggests that SAS may help discriminating species type. Therefore we end with discussing the question 'Is SAS a species-specific communication signal?' in Section 7.4.

In Chapter 2 we introduced a model of courtship song production in which we assume that song amplitude reflects muscle dynamics and neural dynamics. We then test a prediction of our model that SAS signals muscle power in Chapter 3 and we validated this hypothesis in muscle mutants of the *Drosophila myosin light chain gene* (*Dmlc*).[78] However we have not tested the hypotheses of our model that song amplitude reflects muscle and neural dynamics. In Sections 7.3.2 and 7.3.3 we discuss how the hypotheses of our song model that SAS reflects neural and muscle dynamics can be tested in future work.

## 7.1 How does fly movement affect perceived song amplitude?

In our studies (Chapters 3, 4, 5, and 6) we record song with a single microphone that we place below a fly couple that we keep in a chamber with perforated bottom (see Figure 3.2a–b for images of our song recording setup). With this setup we keep fly couples at a relatively constant distance to a microphone, as they can distance themselves only by climbing the chamber wall, which is about 4 mm in height (less than two fly body lengths).

We do not track the distance between male body and microphone which could have allowed us to estimate how fly movement affects perceived amplitude.[31] But measuring the distance between male body and microphone is an approximation only to the distance between sound source and receiver: Males produce songs by extending and vibrating one wing,[25] so the wing is the sound source, rather than the male body. Therefore the best estimate for distance between sender and receiver is the distance between a male's wing and a female's ear. However traditional trackers do not track fly wings;[135] this is a limitation of such trackers in controlling for distance between sender and receiver[31] and further tracker development is needed to address this issue.

This tracking software limitation might be overcome experimentally: an obvious approach for estimating how fly movement affects perceived amplitude is to equip a female fly with a microphone and then pair her with a male. The microphone on the

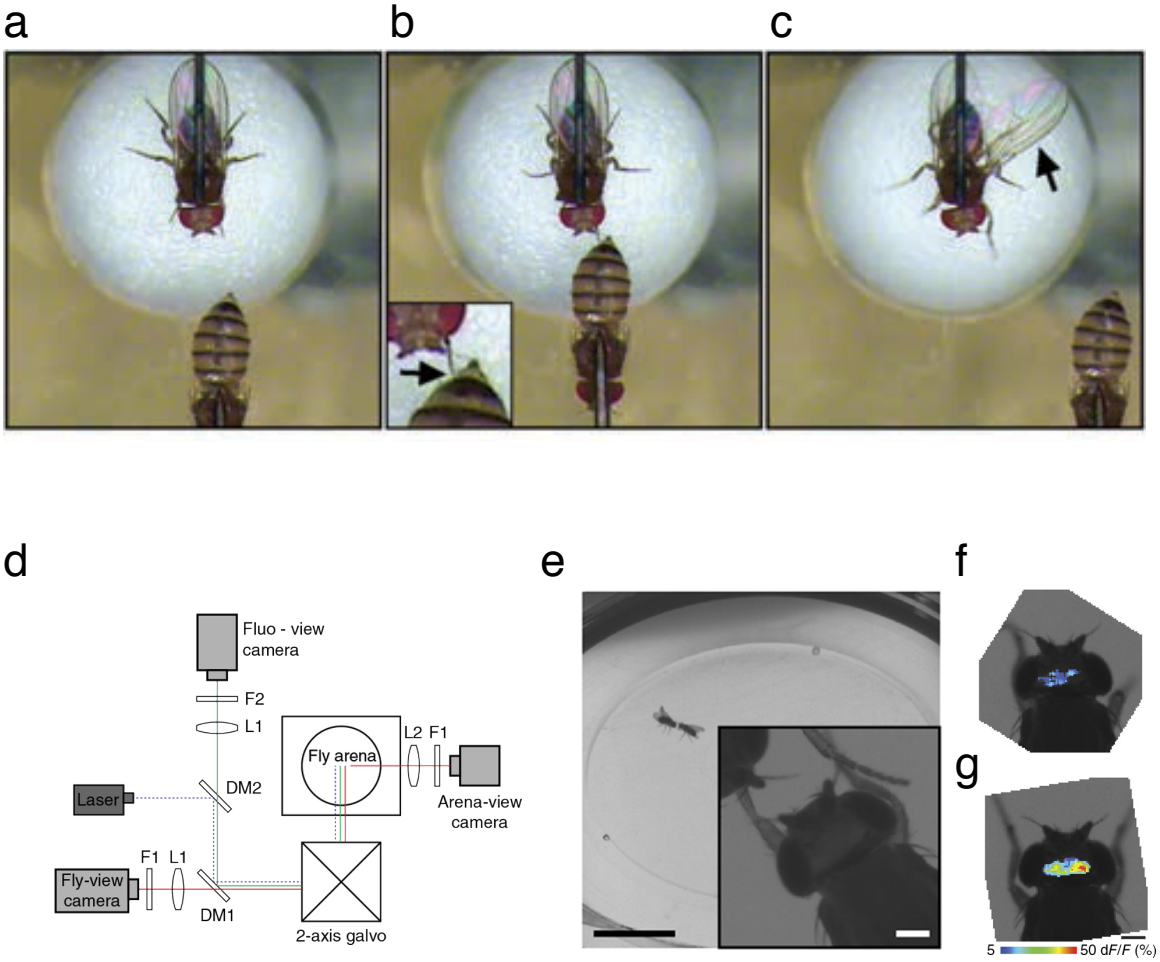



**Figure 7.1:** Setups for studying courtship in *Drosophila*.
(**a**) A male fly is tethered to a pin and placed on a styrofoam ball that is floating on air. The female is presented in front of the male's head without inducing a discernible reaction in the male. (**b**) The female is touched once to the foreleg tarsus of the male (arrow). (**c**) After the contact, the male follows the female and extends a wing on the side to which the female is presented (arrow). (We took images and descriptions in **(a)**, **(b)** and **(c)** from.[47]) (**d**) Schematic of the galvanometer mirror-based tracking system for fluorescence imaging. DM1, DM2 are dichroic mirrors; F1, F2 are optical filters; L1, L2 are lenses. Lines shown in red, green, and dotted blue indicate light paths for the arena backlighting, fluorescence, and laser illumination, respectively. Galvo denotes to galvanometer mirror. (We took the schematic from[18] and adapted the description from.[18]) **(e)** Arena view of a male fly courting a female. The cuticle on the head of the male is removed and replaced with transparent glue as to allow imaging from neurons that control courtship. The male expresses a Calcium indicator in neurons that control courtship; when those neurons are active the intensity of the light they emit increases. The inset show the corresponding fly-view frame with physical contact between the two flies. Scale bars are 10 mm and 200 $\mu m$ in insets. **(f)** Fluorescence intensity in a male brain before a male contacts a female. **(g)** Fluorescence intensity in a male brain after the male contacts a female. (We took images in **(e)**, **(f)** and **(g)** from[18] and adapted descriptions from.[18])

female could then record male song while flies are moving. However a major challenge of this approach will be to engineer a microphone small enough to not obstruct fly movement. Currently the smallest microphones used for recordings of fly song[31,32]

(also see the Methods Section 4.2.1) are five times larger and at least 1000 times heavier than a fruit fly, and flies will be obstructed by this microphone. [1]

An alternative approach to assess how fly movement and distance to a microphone affects perceived amplitude could be to experimentally control distance between flies[31,47,88] (see Figure 7.1a–c). In this approach a live male fly is tethered and then positioned on an airborne styrofoam ball so that his legs touch the surface of the ball and the male fly can walk on the ball.[31,47,88] In this setup a male can be stimulated to court by for example presenting him with a female fly[47,88] (see Figure 7.1a–c). Additionally this '*fly-on-a-ball*' setup allows recording of courtship song.[31] Thus far courtship song in the fly-on-a-ball setup has been recorded at constant distances to a male wing only.[31] One could however measure song at variable distances in a fly-on-a-ball setup by attaching a female and a microphone to a micromanipulator and then varying the distance to a male on the ball. This approach could allow to accurately assess the distance between receiver (female ear) and sender (male wing) while recording fly song.

Although the fly-on-a-ball approach may allow to record what can be perceived by the female fly at various distances to a male, it does not inform what a female actually perceives. The difference between potential reception and actual perception is important: position and angle between flies significantly affects intensity of acoustic signals[44] and thus a female fly may miss even loud song pulses. (For example when a female turns her back on a male, song pulses may not stimulate female ears.[44]) Therefore it is desirable to record song perception during free movement of flies. This may be possible with recently developed '*flyception*',[18] which allows to image brain activity in freely walking fruit flies (see Figure 7.1d–g). Flyception could allow to image activity from auditory neurons[65,71] in a freely walking female while she is courted by a male.

Note however that the resolution for brain imaging in freely moving flies is considerable lower than in head-fixed flies: freely moving flies can be imaged with pixel sizes of about 2240 $\mu m^2$ head-fixed flies can be imaged with pixel sizes of about 7 $\mu m^2$. Therefore when one images head-fixed flies one can better resolve neural structures than when we image freely moving flies. It will be interesting to test whether signals from auditory neurons[65,71,72] can be captured with flyception.[18]

[1] A CMP-5247TF-K particle velocity microphone as used by us and others[32,136] has a diameter of 10 mm which is five times the approximate body length of a fly. A CMP-5247TF-K weighs about 0.75 g[137] and a fruit fly weighs about 0.3 mg when dried;[138] the latter is about 2000 times less than the weight of the microphone. However the weight of a live (non-dried) fruit fly likely exceeds its dried weight.

## 7.2 How do flies auditory process song amplitude structure?

Note that fly ears are described as nonlinear actuators, amplifying low amplitude signals relative to high amplitude signals[67,68] (see Figure 7.2a–b for an illustration of nonlinear actuation in fly ears), which introduces the possibility that differences in song amplitude are compensated by the fly ear, and flies may not perceive amplitude modulation even though fly songs contain it. See for example Figure 4.4 to see a recording of fly song with amplitude modulation. If flies do not perceive amplitude modulations in song, one would expect that flies do not discriminate between a song with constant amplitude (no amplitude modulation) and a song with amplitude modulation. However our work shows that flies discriminate song without amplitude modulations and song with amplitude modulations (see Figure 4.6), which suggests that flies perceive amplitude modulations in song.

Amplitude modulations in song are probably attenuated by fly ears[67,68] and it will be interesting to test how much fly ears attenuate SAS. This can be tested using a Laser Doppler vibrometer, which can measure arista displacement in response to acoustic stimulation.[15,67,68] Thus far, this setup has been used to measure arista displacement in response to sinusoidal stimulation,[68] and SAS is modulation of pulse song, which has a non-sinusoidal structure. [2] However measuring arista displacement in response to pulse song is possible,[139] which should allow testing how fly ears attenuate SAS in pulse song.

There are at least two processes that flies could use to auditory process SAS: spectral decomposition and bandpass filtering.[64,84] Note that the two mentioned mechanisms may co-exist and are not mutually exclusive.

### 7.2.1 Spectral decomposition

Spectral decomposition decomposes a signal (like fly song) into its components; these components can be sine waves of different frequencies. See Figure 7.2c for an illustration of spectral decomposition. When the sine waves are combined one can recreate the original signal. Spectral decomposition allows to gather information on what frequencies are present in a signal. Note that SAS can be viewed as a low-frequency modulation of a higher frequency signal (pulses of about 200 Hz). For example SAS

[2]Note that sine song is also not technically sinusoidal, but is a complex sound. Technically sinusoids are of a single frequency only, and fly sine song can have multiple frequencies.

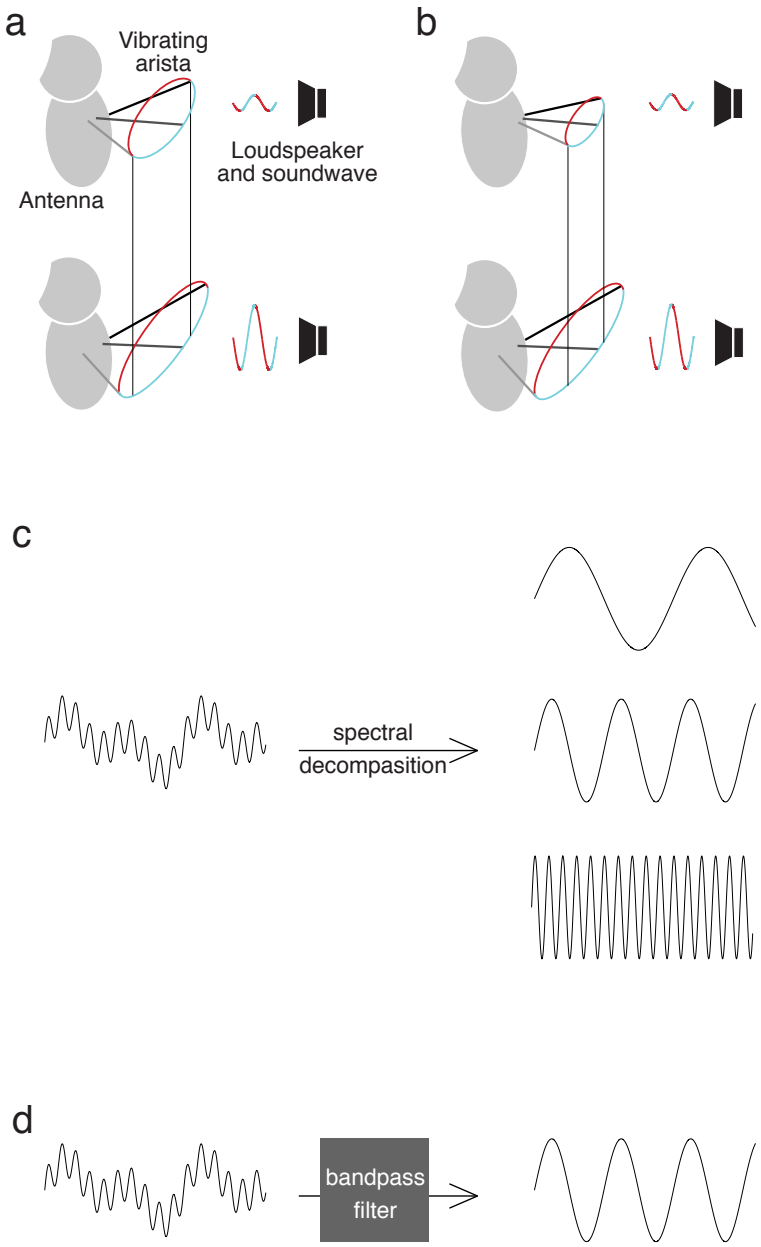



**Figure 7.2:** Auditory processes that may underly SAS processing.
(**a**) Ears of flies are described as nonlinear actuators, when flies are awake and alive.[67] Low amplitude sounds are accentuated. The arista vibrates back (red curve) and forth (blue curve) in response to sound waves. The coloured curves in the sound waves correspond to the coloured curves in arista vibration. (**b**) When flies are dead or anaesthetised their ears become passive and do not accentuate low amplitude sounds.[67] (**c**) Spectral decomposition is a process in which a wave (left side) is transformed into it's underlying sinusoids. SAS can be construed as low frequency modulation of a high frequency signal and SAS low frequency can be extracted by spectral decomposition. (**d**) Bandpass filtering is a process in which a wave is filtered so that sinusoids of a certain frequency bandwidth are extracted. SAS can be construed as low frequency modulation of a high frequency signal and low frequency can be extracted by bandpass filtering.

in *melanogaster* has a repetition rate of about 3 Hz and SAS in *simulans* has a repetition rate of about 4 Hz. Flies could potentially extract information on SAS by spectrally decomposing fly song into its constituents. Indeed mammals use spectral decomposition to process amplitude modulation (comparable to SAS) in signals.[140]

There is some evidence that primary auditory neurons (Johnston Organ neurons; JO) may act as spectral decomposers in signals.[64,65] JO neurons are categorised into five distinct groups of neurons called JO-A, JO-B, JO-C, JO-D, and JO-E.[64] Kamikouchi et al.[65] examined the response of JO neuron groups to three sine-wave stimuli of different frequencies: 0 Hz (static deflection), 19 Hz and 244 Hz (approximate mean frequency of pulse song). Note that the authors measured the Calcium response of neurons; this is regarded as indicator of neural activity.[65,70–72] They showed that—out of the three playback frequencies—JO-A neurons respond most to the 244 Hz stimuli, JO-B neurons respond most to the 19 Hz stimulus and JO-CE neurons respond most to static deflection. (JO-CE denotes the combined response of JO-C and JO-E.) These results[65] suggest that groups of JO neurons differ in what frequencies they respond to. To my knowledge, the work of Kamikouchi et al.[65] is the only published work examining frequency responses of JO neuron groups so far and their results should encourage further work: the frequency response of JO-D is unknown and can be tested[64] and it would be interesting to test responses to frequencies between 0 Hz and about 10 Hz, which are SAS repetition rates we tested with wildtype *D. melanogaster* (see Chapter 4).

Additionally, it would be interesting to examine behavioural responses to playback with SAS when JO neuron groups are silenced. In Chapter 4 we showed that wildtype flies exhibit a robust increased response to their species SAS than to different SAS. If JO neurons extracted repetition rates of SAS, then we would expect that silencing JO neurons would impair the ability of flies to differentiate between playbacks masked with different SAS. Note that silencing all JO neurons renders flies deaf,[65] but flies respond to song playbacks when some JO neurons, but not all JO neurons are silenced.[65] Thus one could silence specific groups of JO neurons and examine the effect on flies' response to playback masked with different SAS.

### 7.2.2 Bandpass filtering

Flies show bandpass-like preferences for their species SAS (see Chapter 4). See Figure 7.2d for an illustration of bandpass filtering. Our behavioural findings raise the question: How can flies bandpass-filter SAS? It has been suggested that crickets use a

process called '*forward masking*'[84] to bandpass-filter amplitude modulations in mating songs and flies may use a similar process to bandpass-filter SAS. Note that the mechanisms for producing amplitude modulations in crickets is likely different to the mechanisms of producing SAS in flies. Crickets produce song by rubbing their wings together (a mechanism called stridulation) whereas flies produce song by extending and vibrating a single wing. It has been suggested that amplitude modulations in crickets reflect the sudden release of an upper wing during wing rubbing,[136] and we suggest that SAS arises from muscle dynamics (see Chapter 3).

Crickets produce amplitude modulated songs[141] and cricket females base mating-decisions on amplitude modulations.[141–143] They exhibit bandpass-like preferences for amplitude modulations typical for their species.[141–143] A process that theoretically can explain bandpass-like preferences for amplitude modulations in crickets is forward masking.[84] Forward masking occurs when nerve cells are activated and fire and then go into a refractory phase that renders nerve cells less sensitive to further stimulation. Present stimulation can mask the effect of future stimulation. The duration of forward masking may depend on the intensity of the present stimulus, with high intensity stimuli (e.g. high amplitude sounds) causing longer masking than low intensity stimuli (e.g. low amplitude sounds).[142] Present stimuli can thus determine how intense future stimuli need to be to activate a neuron.[142] We describe differences in SAS with gain (see Chapter 4), where gain is the relative difference in intensity between successive pulses. The relative difference in intensity of successive pulses can be computed using forward masking.[142]

Forward masking in auditory neurons of crickets is linked to Calcium levels: an increase in Calcium levels is followed by a decrease in neural firing, which suggests that intra-cellular Calcium may decrease neural excitability in these auditory neurons.[144] It would be interesting to test whether auditory bandpass filtering in flies[70–72] is due to a process similar to Calcium-dependent forward masking in auditory neurons of crickets.[144] This could be tested by simultaneously measuring Calcium and voltage levels in candidate auditory neurons in fruit flies, as has been done in crickets.[144] One can thereby investigate the question 'Does Calcium-dependent forward masking explain bandpass filtering of auditory stimuli in flies?'. Candidate auditory neurons for investigating bandpass filtering in *Drosophila* are aPN1,[70] vPN1, and pC1[72] as they have been shown to exhibit bandpass-like activity levels to song characteristics.[70,72] Specifically these neurons respond in a bandpass-like manner to inter-pulse interval (IPI): they are most active in response to playbacks with their species IPI. However, it

remains to be tested whether these neurons respond in a similar bandpass-like manner to SAS.

## 7.3 Does SAS reflect neural and muscle dynamics?

SAS may arise from neural and muscle dynamics (see Chapters 2 and 3) and thus the power development at muscles over time. Our work shows that SAS reflects muscle power in mutants of the *Drosophila myosin light chain* (*Dmlc*) gene (see Chapter 3) but we do not prove that song amplitude signals reflects neural dynamics nor muscle dynamics as we suggest in our model of SAS (Chapter 3). To test whether SAS signals neural and muscle dynamics, song amplitude needs to be recorded together with in vivo neural and muscle dynamics of candidate neurons and muscles that control song amplitude.[31,37,48,51]

### 7.3.1 Is motor neuron activity linked to muscle activity in thoracic muscles?

Two different mechanisms can yield dynamics in muscles that potentially control song amplitude in flies, and it is necessary to examine these mechanisms when investigating the relationship between neural activity, muscle activity and SAS. Muscle activity can depend on (1) motor neuron activation (in twitch muscles as pleurosternal muscles and basalar muscles) or (2) stretch activation [in indirect flight muscles (IFM)].[53]

Twitch muscles contract at rates similar to the firing rates of their respective motorneurons,[20,48,53] which corresponds to one of the hypotheses of our model (see Chapter 3).

However stretch-activated muscles can contract at rates that are different from the firing of their motor neurons.[51] In fact stretch-activated IFMs contract at rates that are 40 times faster than the firing of their corresponding motor neurons.[101] Thus neural firing may not reflect muscle activity, and our model assumption (see Chapter 3) may be wrong for IFMs. However, neural firing rates and IFM muscle activity correlate positively,[101] so our model hypotheses that neural firing rates and muscle activity are positively correlated hold for both types of muscles (twitch muscles and stretch-activated muscles) that potentially control SAS.

### 7.3.2 Does SAS reflect neural dynamics?

A further hypothesis of our model is that motor neuron firing is correlated positively with song amplitude. Motor neuron firing can be recorded from IFMs[31,51,101] and from twitch muscles[48] by inserting a microelectrode into the muscle of interest. Note that microelectrode-recordings from thoracic muscles reflect motor neuron firing as muscles are innervated by a single motor neuron.[31,51,53] To test the hypothesis of our model that song amplitude reflects motor neuron activity, one has to record motor neuron activity at the same time as recording courtship song. Motor neuron activity and song amplitude can be correlated to test our model's assumption.[31] This was done recently for IFMs by Coen et al.[31] who showed that activity of IFM motor neurons correlates positively with song amplitude, which demonstrates that our model assumption holds for IFM. Motor neuron activity of twitch muscles has also been recorded simultaneously with song,[48] but the author did not measure song amplitude, which implies that further work is necessary to test the hypothesis of our model in twitch muscles.

It would be interesting to test whether neural activity in upstream neurons that control song (like P1, as discussed in Chapter 1) is correlated with song amplitude. To test this, one could record neural activity from song control neurons[72] while recording courtship song. To our knowledge there is no published study in which songs have been recorded in parallel with brain-neuron activity, but the fly-on-a-ball setup (see Figure 7.1c–e) can be used for these recordings. In this setup brain activity has been recorded from courting flies[88] and songs also have been recorded,[31] suggesting that it is possible to make both recordings simultaneously. One has to combine them to answer the question: 'Does song amplitude reflect activity of upstream song control neurons?'

### 7.3.3 Does SAS reflect muscle dynamics?

Muscle activity is a function of inner-muscular Calcium levels. Calcium levels positively correlate with muscle tension.[98] Researchers can measure intra-muscular Calcium levels *in vivo* by expressing Calcium indicators in thoracic muscles (see Figure 7.3), which has been done in fruit flies during tethered flight,[20,101] but so far muscle activity has not been recorded from flies while they are singing. Importantly, flies use the same set of thoracic muscles for flying and singing,[48,51] which suggests that the methods for recording muscle activity in flying *Drosophila* may be adapted for recording muscle activity during singing. For this the muscle-activity recording setup[20,101]

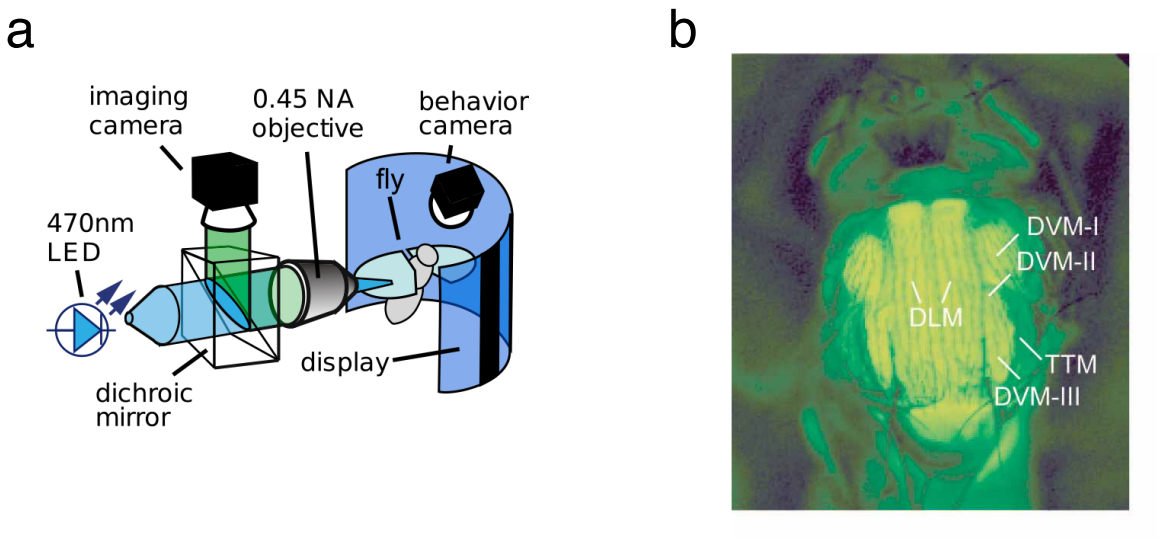



**Figure 7.3:** Imaging muscle activity *in vivo* in *Drosophila*.
(**a**) Setup for recording Calcium. The steering muscles on the right side of the fly are imaged using epifluorescent optics while the fly stabilizes the horizontal position of a vertical stripe in closed loop. (Schematic and description taken from.[20]) (**b**) Calcium indicator expressed specifically in indirect flight muscles (IFMs). (Image taken from.[101])

(see Figure 7.3a) will have to be adapted to stimulate singing in male flies. This can be done by combining the muscle-activity recording setup with a fly-on-a-ball setup (see Figure 7.1c–e).[31,88] A female fly can then be presented to a male fly; this would stimulate him to sing,[88] and the activity of his thoracic muscles can then be recorded *in vivo*.[20,101]

In both setups flies are tethered, which is necessary to minimise movement artefacts when recording Calcium levels.[20,88,101] For recording muscle activity in singing flies, we will have to express a Calcium indicator [3] in flight muscles using for example the UAS/Gal4 system, which is a standard system for expressing Calcium indicators in cells of flies.[20,88,101] The expressed Calcium indicator will emit light (at wavelengths of 535–550 nm), which can be captured with a camera equipped with an emission filter for that wavelength band.[20,88,101] The emission of light of a Calcium indicator depends on Calcium levels in cells. They vary with the activity of cells; for example, the Calcium levels of active muscles are higher than the Calcium levels of inactive muscles.[20,101] This allows researchers to deduce muscle activity based on Calcium-dependent emissions from muscles. Note that cameras can image through a thin cuticle of the fly thorax, thereby penetrating the surface and capturing light

[3] A possible Calcium indicator is GCaMPf.[20] GCaMP stands for a fusion of green fluorescent protein, calmodulin, and myosin light chain kinase.

emissions from muscles without having to open the cuticle.[20,101] This ensures that flies are healthy which is necessary for *in vivo* recordings.

In summary, the hypotheses of our model are testable by using established setups and methods,[20,48,51,72,88,101] which however will need to be combined.

## 7.4 Is SAS a species-specific communication signal?

In Chapter 4 we show that SAS differs between the closely related fly species *D. melanogaster* and *D. simulans* and that flies discriminate behaviourally between the SAS of their species and the SAS of other species (see Chapter 4). Specifically females choose to mate more in response to playback with their species SAS, which shows that SAS affects mate choice. Additionally, the effect of SAS on mate choice is comparable to the effect of IPI on mate choice (see Figure 4.6) and IPI is considered a species-specific communication signal of flies.[29,70,71,145] This suggests that SAS may be a species-specific communication signal. However we compare SAS and responses to SAS only in two species, and more species will have to be tested to thoroughly examine the possibility that SAS is a species-specific communication signal.

What fly species should be tested and why? We can decide what species to test based on at least three criteria: (1) evolutionary relatedness, (2) geographical overlap of populations and (3) species size.

We expect closely related species to be phenotypically more similar than distantly related species. Courtship song is one phenotype that differs between *Drosophila* species[15,36,116] and IPI is one courtship song characteristic that reflects relatedness in some fly species: *D. melanogaster* have an IPI of about 35 ms (see Chapter 4) and closely related *D. simulans* have an IPI of about 48 ms (see Chapter 4), while *D. yakuba*, which is more distantly related to *D. melanogaster* and to *D. simulans*, has an IPI of about 110 ms,[146] so the IPI in these three species reflects their relatedness. It would be interesting to test whether SAS also reflects evolutionary relatedness.

Geographical overlap of species populations can affect the evolutionary pressure to discriminate between species. For example *D. melanogaster* and *D. simulans* habitants overlap, and hybrid offspring of *D. melanogaster* and *D. simulans* are sterile[147] and thus females have a large evolutionary pressure to choose males from their species as mating with their sibling species will result in an evolutionary dead-end. Courtship song can act as signal that affects mating decisions[29,36,116] and we have shown that SAS can render the song of another species unattractive to females (see Chapter 4),

which suggests that SAS may help females to make evolutionary viable mating decisions. We expect that species that share habitats ('sympatric') strongly discriminate species-specific signals as evolutionary pressure can force them to do so.[148] However, species that do not share habitats ('allopatric') may not experience evolutionary pressure to discriminate species-specific signals as they do not meet in the wild. For example *D. yakuba* do not share habitats with *D. melanogaster*, so the two species are allopatric.[16] It would be interesting to test whether females from species that do not share habitats discriminate playbacks masked with each others' SAS. Sympatric species might exhibit stronger discrimination between playbacks masked with each others' SAS than allopatric species.

Body size can differ between fly species[16] and body size correlates with wing size.[58] Wing size can be linked to song amplitude: the larger the wings the louder flies may sing.[31] Additionally large flies likely have larger muscles than small flies and muscle size may affect muscle power, and we show that SAS signals muscle power (see Chapter 3). Thus the body size of flies may affect their SAS and it would be interesting to examine the correlation of SAS to body and wing size of different species.

Differences within a species may also inform whether a signal is species-specific, as one may expect intra-species variation of signals to be smaller than inter-species variation of signals. However, IPI, which is considered to be species-specific,[29,70,71,145] varies considerably across strains of the same species.[32] This is also true for other song characteristics as pulse song frequency and sine song frequency.[32] Thus it is reasonable to ask how the variability of song characteristics as SAS, IPI and song frequencies compares within and between species. To study this we will have to record song of different species as well as songs of different strains of each species. This would allow us to compare the variability of song characteristics between and within species to answer the question: 'Are songs more similar within a species than between species?'.

Note that SAS may act as '*honest signal*'[149] during matings, as SAS reflects muscle power and female flies may prefer to mate with strong males[58] to enhance the fitness of their offspring. However large males which presumably are stronger than small males can hurt females during mating, so large males can reduce female lifespan and reproductive success.[150] Thus females' mate choice may depend on opposing evolutionary pressures: enhancing offspring survival (by mating with strong males) and securing ones own reproductive success (by mating with weak males). Therefore it would be interesting to test whether female flies discriminate the song of weak and strong males and whether females base mating decisions on SAS of weak and strong

males. Note that muscle power affects how much a male sings: strong males sing more than weak males,[43] so SAS is not the only difference between songs of strong and weak males. Thus to specifically assess the potential effect of SAS on mate choice we will have to mask a standard song (e.g. the artificial song we used in our playback experiments in Chapter 4) with SAS of flies with normal or reduced muscle power (see Chapter 3).

## 7.5 Future work

Our work focuses on amplitude modulations in courtship songs of fruit flies. Amplitude modulations are common throughout the animal kingdom: mammals,[9] birds,[3] amphibians,[6] and insects[5,31] modulate amplitudes of their vocalisations. Such amplitude modulations may be adaptations to distance between sender and receiver, termed AMD, which are found in several species.[3,31] However we find that in fruit flies AMD can explain only a small portion of amplitude modulations. We observe amplitude modulations at constant distances between flies and suggest the concept of SAS as explanation (see Chapter 3): SAS describes amplitude modulations as a by-product of neuromuscular activity, which is required to produce sounds. Muscles take time to contract and this may be reflected in amplitude modulations. It would be interesting to investigate how much amplitude variations are explained by AMD in other animals, like birds or mammals (including humans), where AMD has been described.[3] If—as in *Drosophila*—AMD explained only a portion of amplitude variations in these animals, SAS might be a useful concept for the study of amplitude modulations in these animals also. Interestingly muscle activity is correlated with amplitude modulations in the song of starlings,[14] which is an assumption of SAS. As this assumption is true in these song birds, they may be a good model system to pursue the study of SAS.

Note that human speech is amplitude modulated and one reason for these modulations is AMD.[3] To the best of our knowledge it has not been investigated, how much amplitude variability AMD can explain in human speech. If amplitude variations in human speech could not be fully explained by AMD, it would be intriguing to apply SAS to speech processing and examine if muscle activity of muscles involved in speech production (as the diaphragm) correlates with amplitude modulations.

Humans can differentiate amplitude modulations with repetition rates of less than 20 Hz,[102] which is below the frequency range of human hearing. Researchers speculate

that such low frequency modulations are processed distinctly from higher frequency modulations.[102] Interestingly SAS in *D. melanogaster* has a repetition rate of about 4 Hz (see Chapter 4), which is lower than frequencies fly ears are known to respond to.[65] It would be interesting to investigate auditory processing of such low frequency modulations across species.

We began our thesis with the quote "One cannot not communicate"[1] and we suggest in our work that flies cannot not produce SAS. SAS may be a by-product of muscle activity, and it seems to have behavioural relevance for flies. More work is required to elucidate the role of SAS in fly communication, and potentially it's role in communication of other animals.

# Appendix A

# Supplemental list of parameters for mathematical model of fly song production

Table A1 lists all model parameters with their parameter values, respectively the range of parameter values ABC-SMC estimations are based on. Additionally we justify value choice and explain what effect each model parameter has on song, muscle tension or neuronal activity.

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $z$ | (12) | 2ms transformed to $t$ see caption | | The constant $z$ is the duration of an AP in the pulse amplitude pattern neuron. We set $z$ to 2 milliseconds as this is the duration of a normal Action Potential.[151] |
| $\alpha$ | (9) | 0.2 | | The constant $\alpha$ presents the upper boundary of sine song and IPI amplitude. We set $\alpha$ to 0.2 as the maximal amplitude of sine is about 0.2 times the maximum amplitude of pulse song. Our choice is based on visual inspection of song recordings of the Goodwin, Ejima and Simpson lab. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $a$ | (2) | | [0ms, 10ms] transformed to $t$ see caption | Constant $a$ is the temporal difference between the arrival of a signal from command neuron $\Omega$ at the sine and the pulse neuron. We set the lower boundary of $a$ to 0, that is parallel excitation. The upper boundary is with 10ms an ample scope for parameter estimation. Temporal differences in AP conductance are more in the realm of fractions of milliseconds.[151] |
| $d$ | (4) | | [0, 1] | Constant $d$ control decrease of muscle contraction relative to increase of muscle contraction which is set to 1. The lower boundary of $d = 0$ represents the extreme case of no relaxation. The upper boundary of $d = 1$ represents equally fast muscle contraction and relaxation. With boundaries [0, 1] muscles relax slower than they contract [151, p. 678–685]. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $m$ | (10) | | [0, 1] | Muscle tension of $x_i$ lies between [0, 1], hence threshold $m \in [0, 1]$. |
| $j_{\mathrm{s}}$ | (3) | | [0, 250] | Frequency conversion rate $j_{\mathrm{s}}$ determines how muscle tension of the sine muscle is conversed to song frequency. The lower boundary 0 is equivalent to ignoring the activity of the sine muscle. The upper boundary, 250, is equivalent to controlling the maximum song frequency[32] of *D. melanogaster* with the sine muscle. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $\kappa_s$ | (3) | | [0ms,100ms] transformed to $t$ see caption | The constant $\kappa_s$ gives the time, muscle $x_{saf}$ takes to fully contract on continuous excitation. The lower boundary, 0ms, represents instantaneous full muscle contraction, which is a theoretical case. The upper boundary we chose based on experimental data. Josephson reports the tergocoxal muscle in the grasshopper *Neoconocephalus robustus* to take approximately 50 ms to fully contract.[98] Time $\kappa_s$ is conversed to muscle tension increase rate used in Eq. (3) $k_s = \frac{2\times(ln(1-p)+ln(p))}{\kappa_s \times Fs}$, with full muscle contraction $p$ defined as $p = 0.999$. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $g_s$ | (3) | | [0ms,100ms] transformed to $t$ see caption | The constant $g_s$ gives the temporal delay between the time an AP arrives at the neuromuscular junction and the start of the muscles twitch. Theoretically a muscle can start contracting in the instance the AP arrives at the neuromuscular junction, which is represented by the lower boundary of 0 ms. Ashley and Ridgway[152] report small muscles of barnacles to show a delay between application of electrical stimulation and contraction of about 50 ms. We took the double of this reported value as upper boundary. |
| $j_p$ | (3) | | [0, 250] | See the reason for choosing the boundary values of $j_s$. |
| $\kappa_p$ | (3) | | [0ms,100ms] transformed to $t$ see caption | See the reason for choosing the boundary values of $\kappa_s$. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $g_{\mathrm{p}}$ | (3) | | [0ms,100ms]<br>transformed to $t$<br>see caption | See the reason for choosing the boundary values of $g_{\mathrm{s}}$. |
| $\kappa_{\mathrm{pap}}$ | (3) | | [0ms,100ms]<br>transformed to $t$<br>see caption | See the reason for choosing the boundary values of $\kappa_{\mathrm{s}}$. |
| $g_{\mathrm{pap}}$ | (3) | | [0ms,100ms]<br>transformed to $t$<br>see caption | See the reason for choosing the boundary values of $g_{\mathrm{s}}$. |

| Model Parameters | in Eq. | Fixed values | Range of values | Description of parameters and Reasons for value choice |
|---|---|---|---|---|
| $Z$ | (11 – 13) | | [10ms,100ms] transformed to $t$ see caption | The constant $Z$ stands for the duration of one activity cycle of the pulse amplitude pattern neuron $p_{\text{pap}}$. The difference $Z - z$ gives approximately the length of an IPI. The average IPI of *Drosophila melanogaster* is 35ms,[32] while maximum values of IPI in *D. melanogaster* lie at 45 ms. We took double this maximum of IPI and rounded to 100 ms for the upper boundary of value range for $Z$. We chose the lower boundary of Z to be 10 ms, which is a third of the minimal IPI length reported for *D. melanogaster*.[32] |

**Table A1:** Register of all constant model parameters, with the equation(s) they appear in, their fixed values or – if their values are estimated with ABC-SMC – the range of values for estimation and, a description of the parameters and reasons for choosing values. We indicate fixed value parameters with a light grey table cell background. A white cell background indicates values being estimated with ABC-SMC. All of the above values are positive or zero as negative values are not reasonable for the physiological processes described in the model. Some of the values are given in milliseconds (ms) with the note that those values were transformed to $t$. We transformed parameter values in ms to $t$ in three steps: (1) We divided the parameter value by 1000 to transform ms to s. (2) We multiplied the result from (1) with the sampling frequency $Fs$. (3) We rounded the result from (2) to the next lower integer.

## A.1 Parameter Estimation with ABC-SMC

In our ABC-SMC algorithm we compare summary statistics of simulated song and real song. Table A2 lists summary statistics and gives a short description of how we assess summary statistics for real and simulated song.

| Summary statistics | Real Song | Simulated Song |
|---|---|---|
| Carrier frequency | Apply continuous wavelet transform for pulse and multitaper spectral analysis for sine song.[32] | Average simulated song frequency. |
| IPI length | Compute temporal distance between pulses identified with continuous wavelet transform.[32] | Compute temporal distance between pulses identified with function $x_{\text{pap}}$. |
| Ratio sine to pulse | (1) Identify sine song with multitaper spectral analysis and pulses with wavelet transform and heuristic winnowing rules.<br>(2) Sum durations of sine and pulse song.<br>(3) Divide overall duration of sine with overall duration of pulse. | (1) Identify sine and pulse song based on whether $x_{\text{paf}}$ exceeds $m$. Continue with steps (2) and (3) as described for real song. |
| Song bout duration | (1) Identify sine and pulse song.<br>(2) Identify start and end points of sine and pulse bouts.<br>(3) Calculate length of song bouts.<br>(4) Take average across song bout length. | The process is equivalent to what is described for real song. |

**Table A2:** List of summary statistics and description of summary statistics calculation for real and simulated song. We calculated summary statistics for real song with FlySongSegmenter published by Arthur et al.[32] Summary statistics of real and a sample of simulated song are given in Table 2 of the main document.

# Appendix B

# Calcium responses of Johnston Organ neurons to SAS

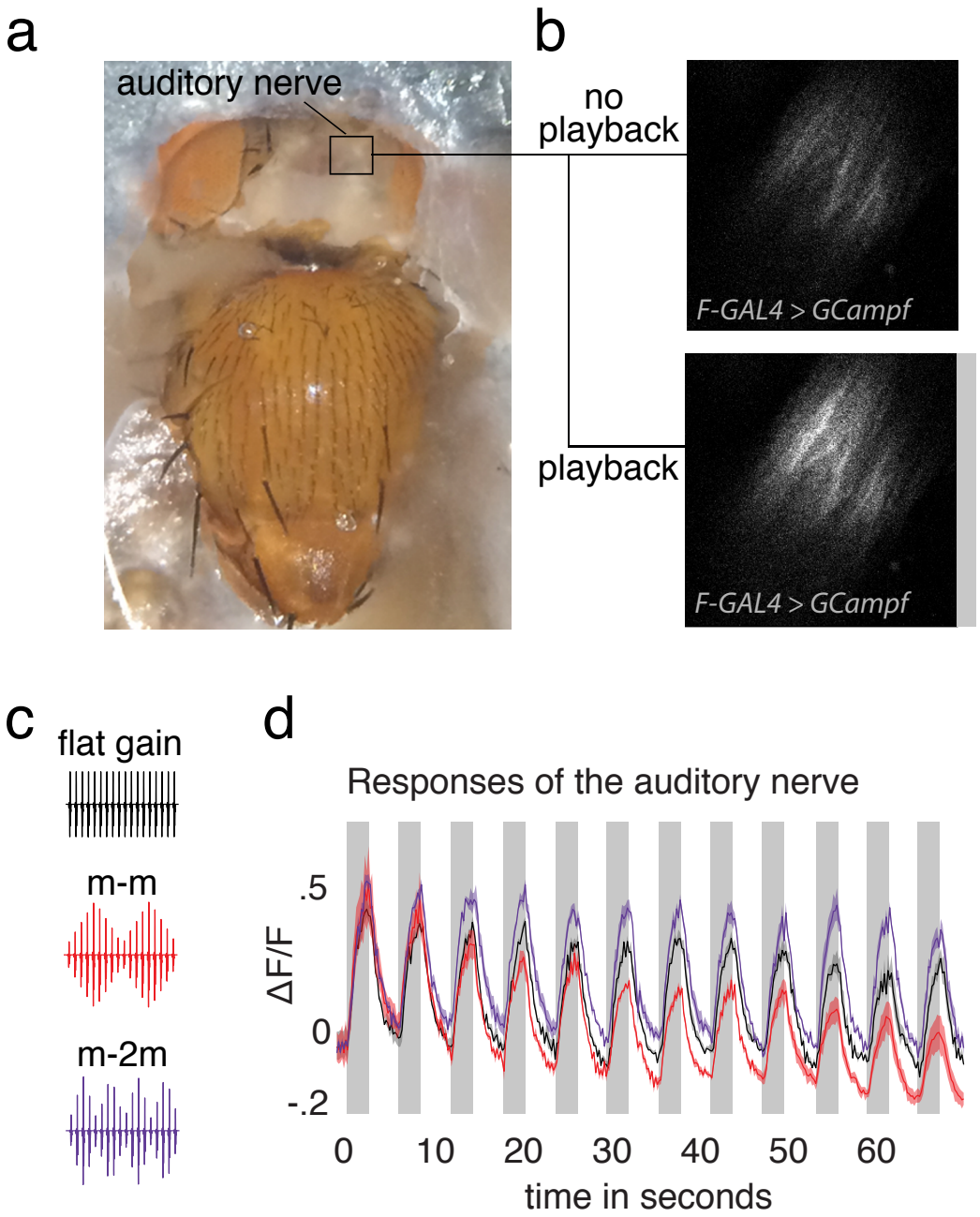



**Figure B.1:** Neural Calcium responses in Johnston Organ neurons in *Drosophila* females in response to auditory stimuli with different SAS.

(**a**) Preparation of *Drosophila* female for *in vivo* imaging of Calcium levels in her auditory nerve. Females express the Calcium indicator *GCampf* in virtually all JO neurons.[65] (Their Genotype is *F-GAL4 > GCampf*.) We remove the cuticle of the head of the female to image from the exposed auditory nerve that we highlight in our image. (**b**) A female auditory nerve exhibits lower Calcium levels when we do not playback song (upper panel) than when we play back song (lower panel). Changes in Calcium levels show as differences in brightness between images. The grey bar on the right side of the lower panel marks a response to playback. (**c**) We used three playback stimuli in our experiment, which differ in SAS only. We controlled for mean amplitude, number of pulses, pulse frequency, and pulse shape of playbacks. Our playbacks consist of trains of artificial pulses with an IPI of 35 ms and a pulse frequency of 200 Hz. (**d**) Calcium responses during playback (of a duration of 125.4 seconds; grey bars) and no playback (of a duration of 188.4 seconds; white gaps). We present playbacks to $n = 4$ females and present each song train 12 times to each female. We repeat this presentation of stimuli two times for each female. We randomise the sequence in which we present stimuli to females. Calcium response traces to stimuli with different SAS diverge over time. The baseline level of the Calcium response to *m-m* playbacks (red trace) decreases over time.

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