A NEUROETHOLOGY APPROACH FOR INSECT SENSES AND BEHAVIOR

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Gil Menda

August 2014

A NEUROETHOLOGY APPROACH FOR INSECT SENSES AND BEHAVIOR

Gil Menda, Ph. D.

Cornell University 2014

Neuroethology is the study of neural basis of natural behavior. I studied learning using sound as a cue in fruit flies and made recordings from the Johnston's organ (JO), I also investigated associative learning in mosquitoes. Finally, I studied Salticid (jumping) spiders to investigate the neural basis of their visual behavior, as well as their acoustic behavior. These three lines of research provided opportunities to learn about ecologically relevant behavior of arthropod models and to learn electrophysiological tools to integrate the study of neural function in behavior.

In terms of the application of these studies, my work on conditioning fruit flies to associate sound may be useful for performing behavioral screens of hearing mutations. Applying the method of bulk conditioning to mosquitoes might be a useful technique for screening “smart” transgenic mosquitoes. Recording from neurons in the central nervous system (CNS) of jumping spiders has never been done, and I plan to share the tools that I developed as well as our new findings (hearing and vision with jumping spider) to benefit the community of researchers who are interested in these fascinating spiders. In the spider work, I have greatly benefited from my collaborations with a

talented team of graduate colleagues, each of whom has made a unique contribution to this work.

BIOGRAPHICAL SKETCH

Gil Menda was born in Israel on July 1971 to Nitza and Igal Menda. Gil has an older sister and a younger brother who died during his military service in 2000. Gil spent most of his childhood outdoors, his parents and grandparents had great influence on his love for nature by taking him outdoors to hike and observe the wildlife. Gil decided at the age of 13 to move to a unique boarding school for environmental studies in the Israeli Negev desert. The school of 'Sde Boker' enhanced Gil's love for bird watching, backpacking, and learning survival in the desert. Those survival skills contributed to Gil's mandatory Israeli 3 year military service in a special combat unit. After the army service he traveled for 1 year, in the Southeast Asia and Australia, following by two-year work as supporting security guard of the Jewish community in Vienna. At that time Gil decided to pursue a Bachelor degree in Plant Protection and Agronomy at the Hebrew University of Jerusalem. During his studies he fell in love with honeybees, and continues his Masters degree in honeybees learning and memory. The death of Gil's younger brother adversely influenced Gil's life, since his brother was very close to him. Once Gil finish his master degree, he decided to take some time off the academia and he opened an apiary with about 200 beehives. He focused on queen breeding, including instrumental inseminations for commercial beekeepers as well as the Israeli Ministry of Agriculture. Once his wife completed her PhD they decided to continue her Postdoctoral work at Cornell University, and they moved to Ithaca, NY with their 2-year-old daughter. Moving to the USA was

challenging, but Gil and his family adjusted quickly to their new life. Gil started to work in the Hoy lab as a part-time technician, and later on Prof. Hoy offered Gil to enroll in a phD program. As a graduate student Gil studied memory and learning of various insects, such as fruit flies and mosquitoes at the beginning of his PhD, those two projects got published and Gil proceed to a different level of analysis with neurophysiology as a tool to work on vision and acoustic of jumping spiders and dragonflies. His work has revealed exciting findings regarding the vision and hearing of this two.

To my brother, Shay Menda, that was a friend more than a brother and was a partner to many adventures. And to my grandfather Shlomo, who introduce me to the love of nature.

ACKNOWLEDGMENTS

First of all I would like to thank my advisor, professor Ron Hoy. I could not have asked for a better role model other than Ron. From the first day I worked with Ron, It felt like I am at home. Ron's personality is admirable; he always makes you feel comfortable and welcome. Ron, as much as he is an amazing person, he is the smartest and most knowledgeable person I have ever met. Each moment around Ron feels like an apprentice next to his master. Throughout the 8 years that I have worked with Ron (2 as technician and 6 as a PhD student), he has always been a phenomenal advisor and a best friend during both the hard and the fun moments. He knew how to put me on the right track, and was very welcoming for my family.

I would like also to thanks my committee, Dr. Laura Harrington, Dr. David Smith and Dr. Cole Gilbert, each of them helped and gave me helpful advice on which direction to take to proceed my PhD. Dr. Harrington and her lab, mainly Sylvie Pitcher, helped me tremendously with the mosquitoes experiments. They hosted me in their lab and made me feel like I am part of their group. I have learned a lot from working with Dr. Harrington, she is also a co-author on my publication in chapter 3 of my thesis, and I am certain we will collaborate on future projects. Dr. David Smith, who is also a co-author on my chapter 3 publication, advised me on learning and memory, he is an expert in his field, and contributed greatly with valuable advice during my PhD. I know we will work more in the future together. Dr. Cole Gilbert gave me very good advices on insect physiology and on my manuscripts, he was also very

supportive in all of the committee meetings and I hope to work with him more in the future.

I would like to thank all the Hoy-lab members, Dr. Pat Rivlin, Dr. Bob Wyttenbach, Dr. Ben Arthur, Paul Shamble, Eyal Nitzani, James Golden and Katherine Walden. Dr. Rivlin is a very good friend and helped me with many of my projects. She is also a co–author on chapter 2. Dr. Wyttenbach is a talented scientist and an amazing critique. He had a major role in publishing chapters 2 and 3, which he co-authored. Paul Shamble, Eyal Nitzani and James Golden are graduate students who are very good friends. They collaborated with me on chapter 4 and we have several currently ongoing and future projects together. We have established together a super-team in which James and Eyal are the computational biology experts, Paul is the spider biology and behavior expert, and Katherine Walden did the behavior experiments. It is a pleasure to work with them in the lab and to plan our research projects together. I would like to thank all the NBB staff, the NBB graduate students, and the NBB Faculty, and mainly to my good friends, Julian, Julie, and Kevin. I would like to thank my family for always supporting me.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION: A NEUROETHOLOGY APPROACH FOR INSECT SENSES AND BEHAVIOR

This dissertation consists of 3 major projects: classical conditioning of fruit flies using sound as a cue(Menda et al., 2011); conditioning mosquitoes to avoid a preferred odor and color as a cue(Menda et al., 2013); and visual perception in the brains of jumping spiders.

Every one of the chapters is in publication format; chapter 2 and 3 are published and chapter 4 is being resubmitted now. Because every chapter has its own publication introduction, I will write an overall introduction for the logic of this work.

The first project was to test if fruit flies can associate sound with a reward, a modality that has not previously been used before in conditioning fruit flies. This project draws on previous experience of conditioning honeybees during my masters degree, and deploying the expertise of the Hoy lab in acoustics. In this project I develop new tools and modify others to develop a partly automatic conditioning apparatus for fruit flies. Our findings that fruit flies can associate sound as a cue are robust so we hope that the science community working on fruit flies will adopt our findings to use as a screening tool for auditory mutations in females and males fruit flies.

Since I was already experienced with conditioning fruit flies, my committee and my advisor suggested moving a step up and conditioning a more complicated insect, the mosquito. Working with Aedes *aegypti* mosquitoes was different because other labs (Alonso et al., 2003)have tried to condition them

with no success such that one author(Alonso and Schuck-paim, 2006) even suggested that this mosquito can't learn. With that in mind I developed a paradigm with much assistance from my committee adviser Dr David Smith. He suggested I try a different approach, and to use a method that called Inhibitory Avoidance (Bermudez-Rattoni and McGaugh, 1991)(McIntyre et al., 2002). In this method you train the animal to avoid an inherent preferred choice, for example a rat's inherent behavior is to be in a dark area, but if you electric shock the rat whenever it is in that area it will avoid it and move to lighted area. I have built a setup that can test many animals at once. Our result is very clear and it shows that Aedes *aegypti* learned to avoid an innately preferred choice (dark surface wall) and remain on a white and bright surface. In this experiment we tested also for multimodal learning, in which we added the component of odor to the vision component. Our results support that adding the odor creates a positive effect on the learning.

During my first 4 years of my PhD I was a TA in a neurophysiology lab course and my knowledge and expertise in neurophysiology got to a level that I could use it as a tool for understanding sensory modality. My PI Dr Ron Hoy took me under his wing, and advised me on different approaches of recording from neurons and I developed a technique to record from the miniscule nervous system of small animals. Dr Hoy challenged me to try to record from Jumping spiders, as a first step, he has trusted that I have the capability to do so. Recording from spiders Central Nerves System (CNS) is known to be a big challenge and until today it hasn't been done by anyone. I used an approach that is not common; I used an old drawing from David Hill (Hill, 1979)

to develop a "landscape" of the spider CNS , with that I could plan the location I would like to record from. Until now many people who have tried to record from J. spider CNS, by intracellular methods using sharp glass electrodes. To do so they had to open the spider head to visualize the CNS (since this electrode is very fragile), by doing so spiders bleed out and die. My approach was to mark the location above the vision central body " bridge" and to insert a very sharp tungsten electrode using landmark placement on the cuticular surface. Once the electrode penetrated the body its hemolymph resealed the hole from the needle. Then I followed by inserting the tungsten micrelectrode for recording. I have been able to hold the same neural unit for many hours and the spider could survive for up to 72 hrs. These recording methods opened up many new possibilities for testing jumping spiders. For example we have recruited undergraduate students to the project and have also recruited grad student collaborators who have expertise in vision and in computational methods. This spider project has yielded many interesting results and opens very interesting future projects with spiders and other invertebrates.

REFERENCES

Alonso, W.J., Schuck-paim, C., 2006. The "ghosts" that pester studies on learning in mosquitoes: guidelines to chase them off. Med. Vet. Entomol. 20, 157–165. doi:10.1111/j.1365-2915.2006.00623.x

Alonso, W.J., Wyatt, T.D., Kelly, D.W., 2003. Are vectors able to learn about their hosts? A case study with *Aedes aegypti* mosquitoes. Mem. Inst. Oswaldo Cruz 98, 665–672.

Bermudez-Rattoni, F., McGaugh, J.L., 1991. Insular cortex and amygdala lesions differentially affect acquisition on inhibitory avoidance and conditioned taste aversion. Brain Res. 549, 165–170. doi:10.1016/0006-8993(91)90616-4

Hill, D.E., 1979. Orientation by jumping spiders of the genus *Phidippus* (Araneae: Salticidae) during the pursuit of prey. Behav. Ecol. Sociobiol. 5, 301–322. doi:10.1007/BF00293678

McIntyre, C.K., Hatfield, T., McGaugh, J.L., 2002. Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. Eur. J. Neurosci. 16, 1223–1226.

Menda, G., Bar, H.Y., Arthur, B.J., Rivlin, P.K., Wyttenbach, R.A., Strawderman, R.L., Hoy, R.R., 2011. Classical conditioning through auditory stimuli in *Drosophila*: methods and models. J. Exp. Biol. 214, 2864 – 2870. doi:10.1242/jeb.055202

Menda, G., Uhr, J.H., Wyttenbach, R.A., Vermeylen, F.M., Smith, D.M., Harrington, L.C., Hoy, R.R., 2013. Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. J. Exp. Biol. 216, 218–223. doi:10.1242/jeb.074898

CHAPTER 2

CLASSICAL CONDITIONING THROUGH AUDITORY STIMULI IN DROSOPHILA: METHODS AND MODELS

Abstract

The role of sound in Drosophila melanogaster courtship, along with its perception via the antennae, is well established, as is the ability of this fly to learn in classical conditioning protocols. Here, we demonstrate that a neutral acoustic stimulus paired with a sucrose reward can be used to condition the proboscis-extension reflex, part of normal feeding behavior. This appetitive conditioning produces results comparable to those obtained with chemical stimuli in aversive conditioning protocols. We applied a logistic model with general estimating equations to predict the dynamics of learning, which successfully predicts the outcome of training and provides a quantitative estimate of the rate of learning. Use of acoustic stimuli with appetitive conditioning provides both an alternative to models most commonly used in studies of learning and memory in Drosophila and a means of testing hearing in both sexes, independently of courtship responsiveness.

* Menda, G. *et al.* Classical conditioning through auditory stimuli in Drosophila: methods and models. *J. Exp. Biol.* 214, 2864 –2870 (2011).

Introduction

Over half a century of research has established the role of sound in *Drosophila* courtship. A male extends one wing and vibrates it at a nearby female, producing a stereotyped pattern of pulse and sine song. Throughout the genus *Drosophila*, these wingbeat songs are species specific in their pattern and harmonic content (Hoikkala and Lumme, 1987; Hoy et al., 1988); *D. melanogaster* is no exception (Bennet-Clark and Ewing, 1967; Bennet-Clark and Ewing, 1969). Females detect this acoustic signal with their antennae (consisting of the arista and Johnston's organ), as has been shown by recordings of sound-evoked field potentials in Johnston's organ and from the antennal nerve (Ewing, 1978; Eberl et al., 2000; Tauber and Eberl, 2003).

Research into learning in *Drosophila* has been going on for about as long as study of courtship. In this time, most work has used aversive stimuli to test learning of chemical cues (e.g. Quinn et al., 1974; Dudai et al., 1976; Tully and Quinn, 1985; Pitman et al., 2009). For example, an odor is presented along with an electrical shock *via* the substrate; after a number of such trials, flies learn to associate the odor with the shock and make avoidance responses when presented with the (previously neutral) odor alone. In terms of classical conditioning, shock is an unconditional stimulus (US), avoidance is an unconditional response (UR) and odor is a conditional stimulus (CS). If associative learning occurs, the UR is evoked by the CS alone after a series of CS–US pairings. Some studies have used appetitive rather than aversive conditioning, with sucrose as the US and proboscis extension as the UR (e.g.

Tempel et al., 1983; Chabaud et al., 2006). When a fly steps in sugar water, its tarsal chemoreceptors trigger a feeding reflex that extends the proboscis, through which it sucks the fluid (Dethier, 1976). This proboscis-extension reflex (PER; Fig 2.1D) is a fixed act, common to many insects, that has been used in studies of olfactory and taste learning in a variety of insects in addition to *Drosophila* (e.g. Nelson, 1971; Bitterman et al., 1983; Daly and Smith, 2000).

Much of the interest in learning and courtship in *Drosophila* is due to its status as a model organism in which mutants can be easily screened. Although many auditory mutants have been identified (Caldwell and Eberl, 2002), there is a limitation in that 'the only known acoustic behavior of fruit flies is their response to courtship songs' (Inagaki et al., 2010). In fact, all current methods of screening for auditory mutants are based on courtship, by testing either the receptivity of females or the tendency of males to court one another when stimulated with pulse song (Eberl et al., 1997; Inagaki et al., 2010). A new method of behaviorally testing hearing in both sexes, independent of courtship, could advance the study of hearing in *Drosophila*.

In the present study, we employed an appetitive conditioning protocol using sugar water as a reward, a non-courtship sound as a neutral stimulus and proboscis extension as an indicator of learning. We also recorded from Johnston's organ to verify that our stimuli were audible. To our knowledge, no prior studies of learning in *Drosophila* have used acoustic stimuli and only a few have used appetitive conditioning.

Materials and Methods

Training experiments

Protocol

Training and testing were done according to the timeline in Fig 2.1 Each fly was given six training trials. In 'paired' trials, flies were rewarded with 5 s access to sucrose (1 mol l^{-1} solution) 5 s after the onset of a 10 s sound stimulus. In 'unpaired' trials, sucrose was presented 30 s after the end of the sound, while sucrose was never presented in 'no-reward' trials. In all three types of trial, PER strength was rated during the first 5 s of sound stimulation, before the onset of any reward, and flies were presented with water 120 s after the sound stimulus to prevent dehydration and to wash off any sucrose remaining on the tarsi. Water presentation was isolated from sound stimulation by 2–3 min before and after, making it unlikely to affect training. The six training trials were followed by two trials testing for retention, one at 15 and one at 25 min after training, in which only the sound was presented and PER strength was rated.

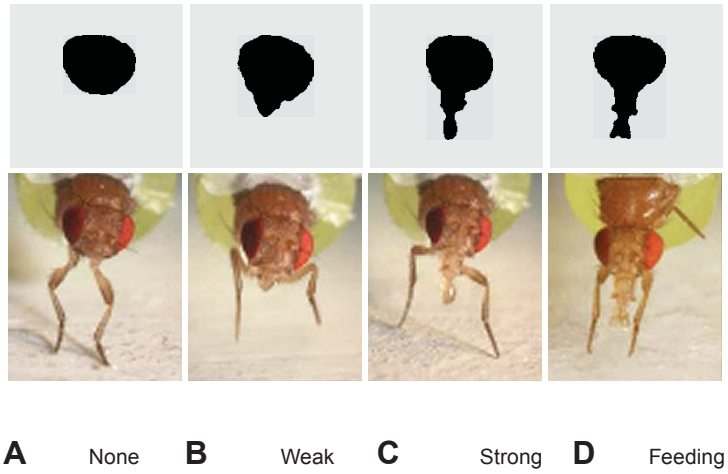


Fig. 2.1. Proboscis extension. When the foreleg tarsi touch sucrose solution, the proboscis reflexively extends to feed as shown in D. With lower levels of excitation, the proboscis may extend only partially (B,C) or not at all (A). Responses to sound were rated as none (A), weak (B. <50% of full extension) or strong (C, ≥50% of full extension).

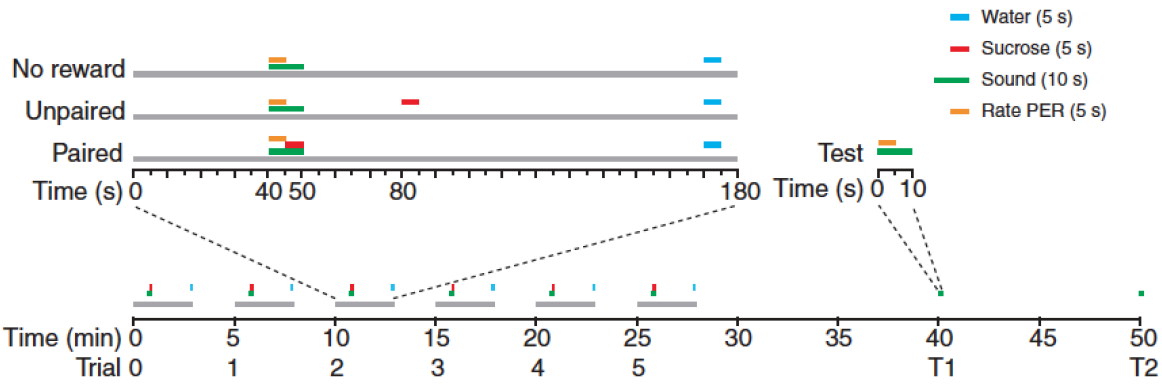


Fig. 2. 2.Training schedule. There were three experimental groups: paired, unpaired and no reward. Each group experienced six training trials (0–5) with a 5 min inter-trial interval, followed by two retention tests (T1 and T2) occurring 15 and 25 min after training. In the paired condition, a 10 s sound stimulus overlapped with a 5 s sucrose reward; in the unpaired condition, presentation of sucrose was delayed by 30 s; in the no-reward condition, sucrose was never given. In all cases water was given 120 s after sound offset. PER, proboscis-extension reflex.

Apparatus

The training device (Fig 2.3.) was built on a rotating 16 cm diameter kymograph drum (Bird Kymograph no. 70-060; Phipps & Bird Inc., Richmond, VA, USA), based on published designs (Vargo et al., 1983; Holliday and Hirsch, 1986; Brigui et al., 1990). The drum rotated fully in 5 min, presenting sound, sucrose and water on the schedule shown in Fig 2.2. The sound stimulus was activated when a magnet on the drum moved past a reed switch 1 cm away from the drum. Closing the switch made no audible sound and did not transmit vibration to the drum. Sucrose solution and water were delivered from two different 8X160 cm strips of filter paper (Whatman no. 2300 916) mounted on the drum, fed by reservoirs on top of the drum. Three flies were tested at a time, each one loaded into a pipette tip and placed with its foreleg tarsi in contact with the drum. The three fly heads filled the frame of a video camera for later analysis.

Stimuli

The conditional stimulus was a 10 s, 400 Hz tone (DynaScan Corp. 3011 function generator; Ivine, CA, USA) broadcast through a 16 cm paper cone woofer and directed at the flies through a plastic funnel, with the 24 mm opening of the funnel 20 mm from the flies, placing flies in the near field. To avoid transmission of vibration to the rotating drum, the speaker was held by a stand not attached to the drum. Intensities were calibrated in dB SPL (re. 20 mPa) with a Brüel & Kjær type 2209 sound level meter with a type 4138 1/8 in microphone in the location that would be occupied by the

central fly. Three intensity levels were used, 65 dB SPL (quiet – just above auditory threshold), 85 dB SPL (moderate – near the natural level of courtship song) (Bennet-Clark, 1971) and 108 dB SPL (loud). All three training conditions, paired, unpaired and no reward, were tested at each of the three intensities.

Response

Proboscis extension is an unconditional response when the fly's tarsi contact sucrose solution (Nelson, 1971; Médioni and Vaysse, 1975; McKenna et al., 1989). All training and test sessions were recorded on video for later frame-by-frame analysis. PER strength was categorized as no response, weak response or strong response (Fig 2.1.) (Chabaud et al., 2006). Rating was carried out by two observers, at least one of whom did not know the training protocol in use.

Subjects

Three-day-old virgin female *D. melanogaster* (Canton-S-5 strain) (McKenna et al., 1989) were taken from our laboratory cultures. They were starved for 24 h before testing to increase their motivation to feed.

Statistical analysis

In Pavlovian conditioning, learning is an increase in response over time when a stimulus predicts a reward (paired trials), compared with a constant response over time when stimulus and reward are uncorrelated (unpaired

trials). To test whether learning occurs, we used a parametric statistical model in which differences between paired and unpaired groups and the change in response within each group over time are integrated into a logistic regression model:

$$\text{Test: } \log\frac{P(t)}{1-P(t)} = \alpha + \beta_T t \quad \text{Control: } \log\frac{P(t)}{1-P(t)} = \alpha + \beta_C t \,, \quad (1)$$

where $P(t)$ is the probability that a fly responds at trial t (t=0 being the initial trial). These equations describe sigmoidal growth curves for probability of response as a function of trial number. The rate of growth is reflected by the regression coefficients b_T (for the test, paired condition) and b_C (for the control, unpaired condition), while the baseline response is reflected by the intercept, a.

Consequently, we can ascertain whether learning occurs using just three statistical tests. First, the log-odds of a response at t=0 are equal to a for both groups. Thus, we test whether the initial response rate is the same for the paired and unpaired groups, using a test for the difference between two proportions or a related procedure such as Fisher's exact test. We call this test T_0 and its null hypothesis $H_0(0)$. Next, any change in response rate over time should be monotonic, as the log-odds are linear functions of t with slopes b_T and b_C for the paired and unpaired groups, respectively. In particular, if the stimulus induces learning, there should be an increase in the response rate of the paired group (i.e. the regression coefficient b_T is positive) but not of the control group (i.e. b_C is zero or less). Thus, our second (T_1) and third

($T2$) statistical tests concern the hypotheses:

$$H0(1) : \mathrm{bC} \leq 0 \; vs \; HA(1) : \mathrm{bC} > 0 \text{ and } H0(2) : \mathrm{bT} \leq 0 \; vs \; HA(2) : \mathrm{bT} > 0 \,. \quad (2)$$

Rejection of $H0(2)$ along with failure to reject $H0(0)$ and $H0(1)$ would be evidence of Pavlovian learning.

Because three tests are performed, the significance level for each test must be adjusted to ensure that the overall experimental Type I error is controlled at a level of 0.05. Using the conservative Bonferroni procedure, hypotheses $H0(0)$, $H0(1)$ and $H0(2)$ are tested at the reduced level $0.05/3=0.0167$. To account for the fact that responses across trials within each fly are dependent on each other, estimates of the regression coefficients (i.e. learning rates), their standard errors, and P-values used in tests of $H0(1)$ and $H0(2)$ are obtained using the technique of generalized estimating equations (GEE) (Zeger and Liang, 1986; Verbeke and Molenberghs, 2001). Using GEE rather than likelihood-based methods ensures that the standard error estimates for regression coefficients reflect the dependence of the responses recorded for each fly across the series of trials, thereby avoiding inappropriate assessments of statistical significance.

Physiological recording

As an independent test of stimulus audibility, we recorded from Johnston's organ. Recording and stimulus calibration were done as previously (Arthur et al., 2010). A fly was dorsally tethered with paraffin and positioned such that the plane of the arista was parallel to sound waves emanating from a speaker.

A tungsten electrode was inserted in the second antennal segment (Johnston's organ) and the Bayesian QUEST procedure (Watson and Pelli, 1983) was used to adaptively quantify thresholds to a 400 Hz stimulus. Thresholds to periodic oscillations at the fundamental and second harmonic of the stimulus frequency were tracked in parallel (King-Smith et al., 1994), with a stimulus deemed above threshold if the response was greater during the 0.5 s stimulus presentation than during an equal amount of time immediately preceding it. The customary Weibull function was used as the assumed neurometric function with the threshold criterion set to 75%, the slope (b) to 0.05 based on pilot data, the probability of failure at infinite intensity (d) to 0.01, and the probability of success at negative infinity (g) to 0.5. To facilitate validation of the estimated slope parameter, each fly was used in ≥200 trials, with a uniform random intensity within the 55–95% range of the current estimated threshold for each trial (supplementary material Fig. S2.6.). The final threshold estimate was taken to be the mean of the posterior density function when its standard deviation dropped to 3 dB. Immediately after Johnston's organ recordings were concluded, control recordings were conducted in the contralateral eye of each fly to check for stimulus artifact due to coupling with the speaker. Both antennae were removed for control recordings because attenuated potentials from antennae may be recorded throughout the head, as we and others have observed in mosquitoes (Wishart et al., 1962; Arthur et al., 2010). In all cases the reference electrode was placed in the thorax through a coxal stump following removal of a leg.

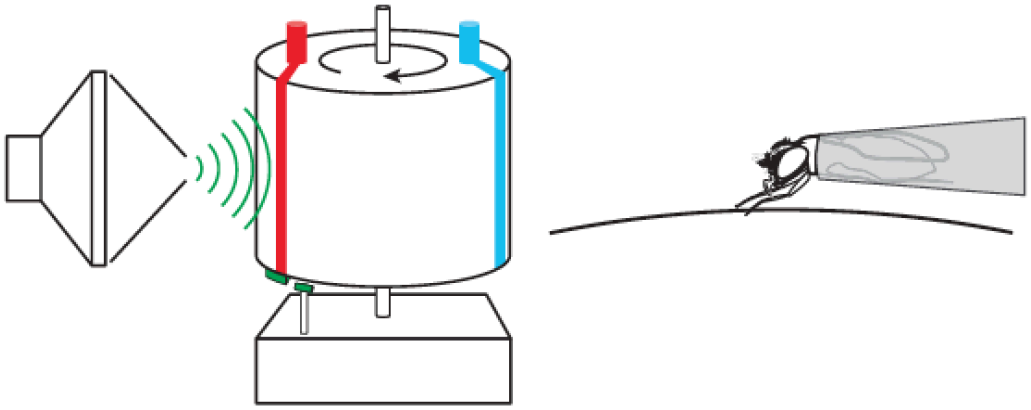


Fig 2. 3. Training apparatus. Flies were mounted in plastic pipette tips and held with their foreleg tarsi touching the surface of a rotating kymograph drum. Rotation of the drum controlled the presentation of sound (green), sucrose (red) and water (blue) according to the schedule shown in Fig. 2.2

Sound intensity and calibration

Because antennae respond to the particle-velocity component of sound (Bennet-Clark, 1971), intensities ought to be calibrated with velocity microphones and presented in dB SPVL (re. 50 nm s^{-1}). However, measurement of particle velocity is difficult in confined spaces; indeed, both velocity and pressure can vary considerably over small distances in echoic environments. Most studies of *Drosophila* hearing and courtship take place in small and echoic chambers (e.g. Eberl et al., 1997; Inagaki et al., 2010), with calibration microphones often placed near but outside the chamber. Some use pressure microphones, some use velocity microphones, and some give no calibration detail at all. As a result, it is difficult to compare intensities across studies.

Calibration of stimuli for learning was done as described above with a pressure-sensitive microphone at the central fly position. We subsequently mapped the sound field between the funnel exit and the kymograph drum with both a pressure microphone and a pressure-gradient (particle-velocity) microphone as detailed below. Intensity was highest at the center of this space, dropping off at the edges and near the drum, with velocity varying more than pressure. At our moderate intensity level, for example, at the surface of the drum we measured 85 dB SPL and 95 dB SPVL at the location of the central fly, and 82 dB SPL and 90 dB SPVL at the edge of the funnel; moving the microphones away from the drum and toward the center of the funnel exit, we measured 88 dB SPL and 109 dB SPVL. The nominal values we report came from the center near the drum; intensities experienced by the three

flies would vary somewhat by position and possibly by trial. While it is unlikely that intensities were precisely 65, 85 or 108 dB SPL for each fly in each trial, it is certain that the 108 dB stimulus was louder than the 85 dB stimulus, which was louder than the 65 dB stimulus.

Without the constraint of putting flies in a training apparatus, physiological recording took place in a far cleaner acoustic environment. In an anechoic far field, dB SPL and dB SPVL are numerically equal (Bennet-Clark, 1971) because a pressure of 20 mPa (0 dB SPL) corresponds to a velocity of 50 nm s^{-1} (0 dB SPVL). We calibrated in dB SPVL using a pressure-gradient microphone (Knowles NR-23158) as described previously (Arthur et al., 2010) and in dB SPL using a pressure microphone (Brüel & Kjær type 4138). The results confirm that our recording location was in fact anechoic and far-field at 400 Hz.

RESULTS

The results of the training study are shown in Fig 2.4. For the purpose of analysis, weak and strong PER responses were lumped to create simple response and no-response categories. Results in the no-reward condition were indistinguishable from those in the unpaired condition, so our analysis considered only the paired and unpaired trials. It is clear that flies in the unpaired condition did not learn to associate sound with sucrose: the proportion responding stayed at the initial level throughout. Flies in the paired condition showed an increase in response probability as trials progressed, indicating that they associated the sound with the sucrose reward. Statistically, these observations are borne out at each intensity level by the three tests described above. The initial response probability did not differ between paired and unpaired groups (Fisher's exact test: P=0.45 at 65 dB, P=0.49 at 85 dB, P=0.24 at 108 dB), regression coefficients of unpaired trials are not significantly greater than zero (GEE Z- test: P=0.43 at 65 dB, P=0.13 at 85 dB, P=0.30 at 108 dB), and regression coefficients of paired trials are positive (GEE Z-test:

P=0.0167 at 65 dB, P=0.0006 at 85 dB, P<0.0001 at 108 dB). Thus,

the results meet the standards of learning as described by our logistic regression model. In the two test trials, responses remained high, showing that the association between sound and sucrose was retained 15 and 25 min after the end of training.

To test whether learning required the auditory modality, we conducted a set of paired trials at 108 dB with 14 flies in which the antennae were

ablated. These flies did not show an increase in response as trials progressed (GEE *Z*-test: b_T=–0.18±0.12, mean ± s.e.m., *P*=0.11; data not shown).

It is clear from Fig 2.4. that stimulus intensity affected the proportion of flies responding in all trials, in both paired and unpaired groups. As sound intensity increased, the proportion responding decreased (chi-square test: *P*<0.0001). However, the slope of the logistic regression (b_T) did not vary significantly with intensity.

Recordings from Johnston's organ (Fig 2.5.) show that all stimuli were audible. The response consists of a phasic negative deflection of the baseline followed by a tonic positive deflection. In addition, the field potential oscillates at the frequency of the stimulus and its harmonics. Our Bayesian threshold search was based on the oscillatory potential; *post hoc* analysis of the baseline shift showed its threshold to be higher than that of the oscillatory potential. In one fly, the baseline shift was not phasic but was a tonic negative deflection (supplementary material Fig. S 2.7.) as in *Aedes* mosquitoes (Cator et al., 2009; Arthur et al., 2010). Across all 11 flies tested, thresholds with a 75% criterion were 65±1 dB (mean ± s.e.m.) for the stimulus frequency (F0) and 64±1 dB for twice the stimulus frequency (F1) with a 0.5 s stimulus. These levels are approximately those used as the least intense CS in training experiments. However, these may be conservative estimates of threshold. With longer stimuli, such as the 10 s used in our learning experiments, thresholds could be lower if flies integrate over long periods.

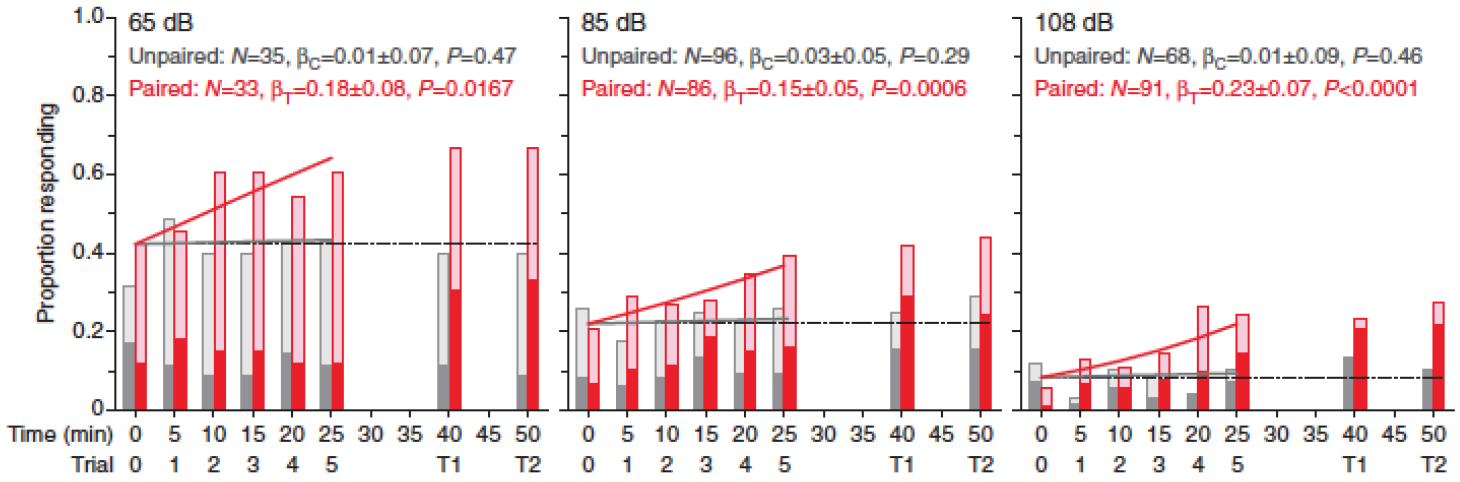


Fig 2. 4. Response to sound during training. Bars show the proportion responding in the paired (red) and unpaired (gray) groups. Dark portions indicate a strong PER; light portions indicate a weak PER. The no-reward group (not shown) was indistinguishable from the unpaired group. Curves show the response probabilities for each group predicted by our GEE model; dashed lines are the no-learning rates (equal to the initial response rate). For each intensity and test condition, we report the number of flies (N), the value and standard error of the GEE regression slope parameter (bT for the test paired condition; bC for the control unpaired condition) and the P-value for the regression parameter. The last two points (T1 and T2) are tests of retention in which only the sound was presented.

DISCUSSION

Appetitive conditioning with acoustic stimuli

Our results and analysis show that female *Drosophila* learned to associate a tone with a sucrose reward. While *Drosophila* has long been a model system for the study of learning and memory, most previous work has used chemical stimuli as the CS and aversive stimuli, generally electrical shock, as the US (e.g. Quinn et al., 1974; Dudai et al., 1976; Tully and Quinn, 1985; Pitman et al., 2009). In contrast, we used an acoustic CS and a rewarding US. The prior study most similar to ours was by Chabaud and colleagues, who examined appetitive conditioning with an olfactory CS (Chabaud et al., 2006). Their wild-type flies started from a baseline of 25–40% PER, increasing to 60–75% PER after five trials, similar to the baseline and change we found in the 65 dB group.

Sound is neutral in the context of feeding but salient in the context of courtship. We chose 400 Hz as the CS in the hope of finding an auditory stimulus that was neutral but audible. This frequency is sufficiently far from that of the sinusoidal component of courtship song (160 Hz) (Wheeler et al., 1988) that it is unlikely to be perceived as courtship, but likely to be within the audible range for insects that hear with their antennae. Recordings from Johnston's organ verified that the CS was above threshold.

Given our assumption that 400 Hz is neutral, neither attractive nor aversive, the effect of intensity on proboscis extension is puzzling. While the 65 dB group started from a baseline of 40% PER, the 108 dB group started at only 10% PER. Many animals, including insects, respond to loud

sounds with an acoustic startle response that, while not eliciting escape behavior, freezes or disrupts ongoing behavior (Eaton, 1984; Hoy, 1989). This may account for the low PER at 108 dB. Indeed, informal analysis of video collected before each trial found flies in the 108 dB group extending their proboscises, in the absence of stimuli, to an extent approximately equal to that found during the 65 dB trials. Thus, it appears that proboscis extension was reduced by loud sound. Despite its effect on background responsiveness, intensity had no effect on the rate of learning (bT in Fig 2.4), contradicting the usual expectation that rate increases with CS intensity (Davey, 1981). Taken together, these observations suggest that sound intensity can be kept near threshold in learning studies, avoiding startle without slowing the rate of learning. If a lower background response is desired, it can be achieved by increasing sound intensity.

Analysis of learning

Because our statistical tests for learning differ from those commonly used in *Drosophila* learning research, we offer an explanation and comparison with other methods. A logistic model with GEE estimation, hereafter referred to as LGEE, is generally applicable to associative learning. It has been used in several other learning studies; our method is most similar to that recommended by Hartz and colleagues (Hartz et al., 2001) and used by Shafir and colleagues (Shafir et al., 2005). Use of LGEE is motivated by two statistical considerations. First, the logistic model captures the dynamic,

sequential nature of learning in a simple model that permits meaningful tests for time trends within groups as well as for differences in learning rates between groups. Indeed, logistic regression is the most commonly used statistical tool for modeling trends in proportions (Collett, 2002). Second, LGEE uses all of the data collected from each subject on each trial while accounting for the dependence of responses within a subject across trials.

Although established as a learning model in other research communities, the use of LGEE with *Drosophila* is novel. Many studies simply report the difference between post-training and pre- training responses and compare it between paired and unpaired groups. While this is a valid way to determine whether learning occurs, it provides no information about the rate of learning. Chabaud and colleagues (Chabaud et al., 2006) improved on this by assessing learning as follows. (1) For each trial, employ a chi- square or related test (such as Fisher's exact) to check whether the proportion of responses in the paired group differs from that in the unpaired group. (2) For each group, apply Cochran's *Q*-test to determine whether the proportion responding is constant over time. Thus for six training trials, a total of eight statistical tests are required, six to compare responses between groups in each trial and two to test for constant response in each group. We henceforth refer to this set of procedures as CHIQ.

The CHIQ procedure enforces no preconceived notion of learning, in the form of either trends over time or the direction of differences between paired and unpaired groups. Thus, there is ambiguity in how one detects learning. Must each of the chi-square tests be statistically significant? If

not, which of them must be significant in order to conclude that learning has occurred? The role of Cochran's *Q*-test is unclear when used with the full set of between- group tests. It is not even clear that the test is valid in this context. As originally designed, Cochran's *Q*-test was not meant to evaluate the equality of proportions across trials within a group unless more stringent assumptions are imposed on the probability of response for all subjects in all pairs of trials (Bhapkar, 1973; Somes and Bhapkar, 1977). A further drawback of CHIQ is the need to adjust significance levels for the larger number of tests. For an overall type I error of 0.05, each of the eight tests that would be required for our data would have to be done at a level of 0.05/8=0.00625, as opposed to the 0.0167 required for LGEE. This, combined with the fact that none of the tests used in CHIQ are directional, greatly increases its conservatism in assessing learning. The associated price is a loss of statistical power, possibly severe, for detecting learning when learning in fact exists.

LGEE equates learning with a monotonic increase in the proportion responding over a series of trials in the paired group but not in the unpaired group, while CHIQ detects any kind of change in response profiles, either between or within groups. In theory, this gives CHIQ flexibility, trading loss of statistical power for greater robustness. For example, the LGEE assumption of monotonicity could be violated by satiation, although this is not a concern with a relatively small number of trials and a well-designed experiment. In principle, however, we argue that true learning must manifest itself, at least initially, in non-decreasing (if not monotonically

increasing) responses, and the primary goal should be detection of that directional trend. For this purpose, LGEE is powerful and robust. Furthermore, it would be easy to extend the logistic model to allow both non-decreasing and non-monotonic trends, although as with CHIQ, adding such flexibility complicates the definition of learning.

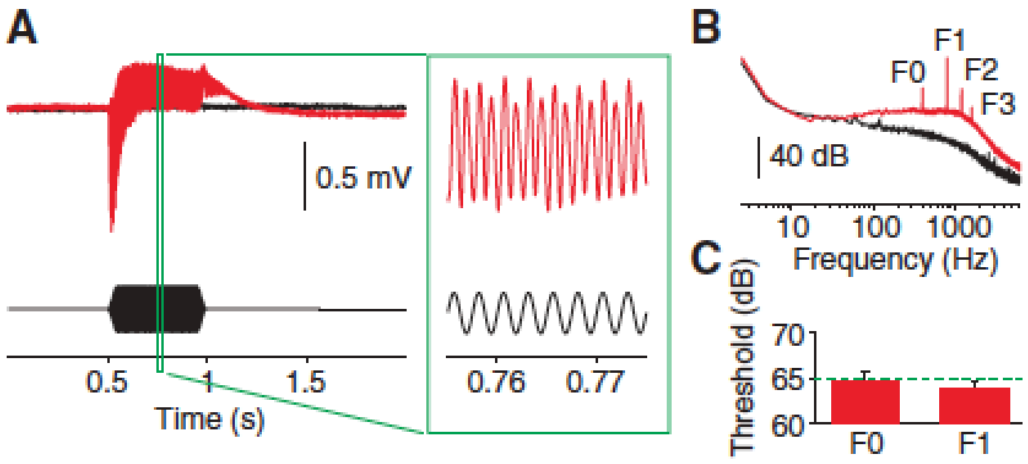


Fig 2.5. Field potentials in Johnston's organ. (A) Sample response to 400 Hz in a female fly (averaged over 85 repetitions). Control recording from the eye is overlaid in black; stimulus is shown below. Recordings were made over a wide range of intensities; this example is for 107 dB, where the high signal-to-noise ratio made the response clearly visible.

(B) Spectral analysis of the sample response in A. Labels F1 to F3 indicate harmonics of the 400 Hz fundamental frequency, F0.

(C) Response thresholds (75% criterion) averaged across 11 females. Thresholds are shown separately for components of the response at the stimulus frequency (F0) and twice the stimulus frequency (F1). The dashed line indicates the lowest intensity used in the learning experiments.

Auditory responses

Recordings from Johnston's organ confirm that the auditory stimuli in learning tests were above sensory threshold. Our threshold of 64–65 dB SPVL in females is close to the 72 dB SPL threshold found behaviorally in males (Eberl et al., 1997). The oscillatory component of the evoked field potential was qualitatively similar to that recorded in mosquitoes (e.g. Tischner, 1953; Wishart et al., 1962). Most baseline shifts were phasic, as described for *Culex* mosquitoes by Warren and colleagues (Warren et al., 2009), but one fly showed a sustained deflection such as we found in *Aedes* mosquitoes (Cator et al., 2009; Arthur et al., 2010). Atypical recordings from one of 11 flies might be dismissed as damage during preparation, but this fly had a normal threshold and its responses were consistent and similar to those found in other species. We suspect that the nature of the baseline shift varies with electrode placement in a heterogeneous population of scolopidia (Kamikouchi et al., 2006; Kamikouchi et al., 2009); future studies could systematically vary placement to test this.

Recording from Johnston's organ in *Drosophila* is not difficult, and we suggest that future studies using auditory stimuli in associative learning assays be combined with recordings to narrow down the anatomic location of deficits. Similarly, if auditory mutants are used in associative learning assays, it is important to test them with other learning assays to control for possible pleiotropic effects on learning.

CONCLUSIONS

Aside from the nice historical coincidence that Pavlov's original model works for flies as well as for dogs, there are good scientific reasons to develop diverse learning models for *Drosophila*. For example, the learning mutants *dunce* and *rutabaga* fail to learn odors in a shock-avoidance protocol but learn normally in an appetitive task (Tempel et al., 1983), while aversive and appetitive learning of odors take place through different neural and biochemical pathways in *Drosophila* larvae (Honjo and Furukubo-Tokunaga, 2009). Learning mutants may act differently not only in different protocols but also with different CS modalities. In general, a greater variety of tests would allow a finer parsing of the array of learning mutations.

In principle, our methods could be adapted to test hearing in both sexes independently of courtship, facilitating the discovery of new auditory mutations. While screening for auditory mutations in males is well established (Eberl et al., 1997), there is no known way to screen auditory mutations in females beyond testing for courtship receptivity (Inagaki et al., 2010). Given that factors other than hearing are likely to affect courtship in both sexes, a test of hearing that does not rely on courtship could be valuable; our method seems to be the only such test available at present.

Finally, we urge that researchers in *Drosophila* learning adopt the logistic regression model with generalized estimating equations. It is a more statistically valid means of analysis than those commonly used today. At the same time, it allows different learning mutants to be quantitatively

compared not only in their overall level of learning but also in their rate of learning, which may differ between mutants and could eventually provide insight into mechanisms.

ACKNOWLEDGEMENTS

We thank Dustin Rubinstein for thoughtful comments and discussion, Margaret Nelson for advice on the setup, Kristin Gawera for drawing the fly in Fig 2. 3., and two anonymous reviewers for helpful comments on an earlier version of the manuscript. This research was supported by NIH award 5R01DC103-36 to R.R.H. Deposited in PMC for release after 12 months.

REFERENCES

Arthur, B. J., Wyttenbach, R. A., Harrington, L. C. and Hoy, R. R. (2010). Neural responses to one- and two-tone stimuli in the hearing organ of the dengue vector mosquito. J. Exp. Biol. **213**, 1376-1385.

Bennet-Clark, H. C. (1971). Acoustics of insect song. Nature **234**, 255-259.

Bennet-Clark, H. C. and Ewing, A. W. (1967). Stimuli provided by courtship of male Drosophila melanogaster. Nature **215**, 669-671.

Bennet-Clark, H. C. and Ewing, A. W. (1969). Pulse interval as a critical parameter in the courtship song of Drosophila melanogaster. Anim. Behav. **17**, 755-759.

Bhapkar, V. P. (1973). On the comparison of proportions in matched samples. Sankhya - Indian J. Stat. A **35**, 341-356.

Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (Apis mellifera). J. Comp. Psychol. **97**, 107- 119.

Brigui, N., le Bourg, E. and Médioni, J. (1990). Conditioned suppression of the proboscis-extension response in young, middle-aged, and old Drosophila melanogaster flies: acquisition and extinction. J. Comp. Psychol. **104**, 289-296.

Caldwell, J. C. and Eberl, D. F. (2002). Towards a molecular understanding of Drosophila hearing. J. Neurobiol. **53**, 172-189.

Cator, L. J., Arthur, B. J., Harrington, L. C. and Hoy, R. R. (2009). Harmonic convergence in the love songs of the dengue vector mosquito. Science **323**, 1077- 1079.

Chabaud, M.-A., Devaud, J.-M., Pham-Delègue, M.-H., Preat, T. and Kaiser, L. (2006). Olfactory conditioning of proboscis activity in Drosophila melanogaster. J. Comp. Physiol. A **192**, 1335-1348.

Collett, D. (2002). Modelling Binary Data (2nd edn). Boca Raton, FL: Chapman & Hall/CRC.

Daly, K. C. and Smith, B. H. (2000). Associative olfactory learning in the moth Manduca sexta. J. Exp. Biol. **203**, 2025-2038.

Davey, G. (1981). Animal Learning and Conditioning. New York: Palgrave Macmillan.

Dethier, V. G. (1976). The Hungry Fly: A Physiological Study of the Behavior Associated with Feeding. New York: Harvard University Press.

Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G. and Benzer, S. (1976). dunce, a mutant of Drosophila deficient in learning. Proc. Natl. Acad. Sci. USA **73**, 1684-1688.

Eaton, R. C. (1984). Neural Mechanisms of Startle Behavior. New York: Springer.

Eberl, D. F., Duyk, G. M. and Perrimon, N. (1997). A genetic screen for mutations that disrupt an auditory response in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **94**, 14837-14842.

Eberl, D. F., Hardy, R. W. and Kernan, M. J. (2000). Genetically similar transduction mechanisms for touch and hearing in Drosophila. J. Neurosci. **20**, 5981-5988.

Ewing, A. W. (1978). The antenna of Drosophila as a 'love song' receptor. Physiol. Entomol. **3**, 33-36.

Hartz, S. M., Ben-Shahar, Y. and Tyler, M. (2001). Logistic growth curve analysis in associative learning data. Anim. Cogn. **3**, 185-189.

Hoikkala, A. and Lumme, J. (1987). The genetic basis of evolution of the male courtship sounds in the Drosophila virilis group. Evolution **41**, 827-845.

Holliday, M. and Hirsch, J. (1986). Excitatory conditioning of individual Drosophila melanogaster. J. Exp. Psychol. Anim. Behav. Process. **12**, 131-142.

Honjo, K. and Furukubo-Tokunaga, K. (2009). Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in Drosophila larvae. J. Neurosci. **29**, 852-862.

Hoy, R. R. (1989). Startle, categorical response, and attention in acoustic behavior of insects. Annu. Rev. Neurosci. **12**, 355-375.

Hoy, R., Hoikkala, A. and Kaneshiro, K. (1988). Hawaiian courtship songs: evolutionary innovation in communication signals of Drosophila. Science **240**, 217- 219.

Inagaki, H. K., Kamikouchi, A. and Ito, K. (2010). Protocol for quantifying sound- sensing ability of Drosophila melanogaster. Nat. Protoc. **5**, 26-30.

Kamikouchi, A., Shimada, T. and Ito, K. (2006). Comprehensive classification of the auditory sensory projections in the brain of the fruit fly Drosophila melanogaster. J. Comp. Neurol. **499**, 317-356.

Kamikouchi, A., Inagaki, H. K., Effertz, T., Hendrich, O., Fiala, A., Gopfert, M. C. and Ito, K. (2009). The neural basis of Drosophila gravity-sensing and hearing. Nature **458**, 165-171.

King-Smith, P. E., Grigsby, S. S., Vingrys, A. J., Benes, S. C. and Supowit, A. (1994). Efficient and unbiased modifications of the QUEST threshold method: theory, simulations, experimental evaluation and practical implementation. Vision Res. **34**, 885-912.

McKenna, M., Monte, P., Helfand, S. L., Woodard, C. and Carlson, J. (1989). A simple chemosensory response in Drosophila and the isolation of acj mutants in which it is affected. Proc. Natl. Acad. Sci. USA **86**, 8118-8122.

Médioni, J. and Vaysse, G. (1975). Conditional suppression of a reflex in Drosophila melanogaster: acquisition and extinction. C. R. Sceances. Soc. Biol. Fil. **169**, 1386- 1391.

Nelson, M. C. (1971). Classical conditioning in the blowfly (Phormia regina): associative and excitatory factors. J. Comp. Physiol. Psychol. **77**, 353-368.

Pitman, J., DasGupta, S., Krashes, M., Leung, B., Perrat, P. and Waddell, S. (2009). There are many ways to train a fly. Fly **3**, 3-9.

Quinn, W. G., Harris, W. A. and Benzer, S. (1974). Conditioned behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **71**, 708-712.

Shafir, S., Menda, G. and Smith, B. H. (2005). Caste-specific differences in risk sensitivity in honeybees, Apis mellifera. Anim. Behav. **69**, 859-868.

Somes, G. W. and Bhapkar, V. P. (1977). A simulation study on a Wald statistic and Cochran's Q statistic for stratified samples. Biometrics **33**, 643-651.

Tauber, E. and Eberl, D. F. (2003). Acoustic comunication in Drosophila. Behav. Processes **64**, 197-210.

Tempel, B. L., Bonini, N., Dawson, D. R. and Quinn, W. G. (1983). Reward learning in normal and mutant Drosophila. Proc. Natl. Acad. Sci. USA **80**, 1482-1486.

Tischner, H. (1953). Über den Gehörsinn von Stechmücken. Acustica **3**, 335-343.

Tully, T. and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant Drosophila melanogaster. J. Comp. Physiol. A **157**, 263-277.

Vargo, M., Holliday, M. and Hirsch, J. (1983). Automated stimulus presentation for the proboscis extension reflex in diptera. Behav. Res. Methods Instrum. **15**, 1-4.

Verbeke, G. and Molenberghs, G. (2001). Linear Mixed Models for Longitudinal Data. New York: Springer.

Warren, B., Gibson, G. and Russell, I. J. (2009). Sex recognition through midflight mating duets in Culex mosquitoes is mediated by acoustic distortion. Curr. Biol. **19**, 485-491.

Watson, A. B. and Pelli, D. G. (1983). QUEST: a Bayesian adaptive psychometric method. Percept. Psychophys. **33**, 113-120.

Wheeler, D. A., Fields, W. L. and Hall, J. C. (1988). Spectral analysis of Drosophila courtship songs: D. melanogaster, D. simulans, and their interspecific hybrid. Behav. Genet. **18**, 675-703.

Wishart, G., van Sickle, G. R. and Riordan, D. F. (1962). Orientation of the males of Aedes aegypti (L.) (Diptera: Culicidae) to sound. Can. Entomol. **94**, 613-626.

Zeger, S. L. and Liang, K. Y. (1986). Longitudinal data analysis for discrete and continuous outcomes. Biometrics **42**, 121-130.

Appendix

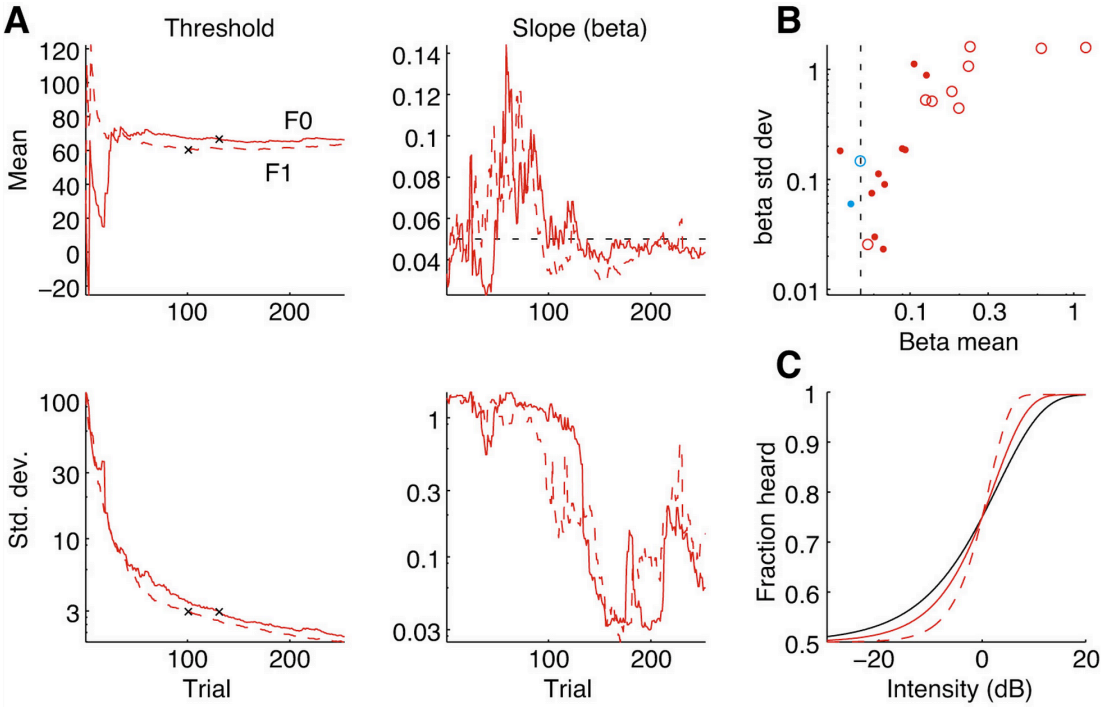


Fig. S 2.6. Slope of the neurometric function. (A) Estimates of the threshold and slope of the neurometric function improved with increasing numbers of trials. The left panels show the mean and standard deviation of the posterior density function calculated with the slope (β) of the Weibull neurometric function set to 0.05 based on pilot data. The right panel show, for the same fly, the mean and standard deviation of the slope value that would maximize the mode of the posterior density function, a measure of goodness of fit. Data for responses at the stimulus frequency (F0, solid lines) are plotted separately from those at the second harmonic (F1, dashed lines). Crosses mark points at which the standard deviation of the threshold estimate drops to 3 dB. (B) For 11 females, estimated slope values for both F0 (open circles) and F1 (filled

circles) were smallest for those that had the smallest standard deviation. Data points shown in A are in blue. The horizontal and vertical black dashed lines in A and B indicate the pilot-derived slope value of 0.05. (C) The Weibull neurometric function with β=0.05, δ=0.01 and γ=0.5 is shown in black. Red curves are for values of β corresponding to the average of the estimated slope values with standard deviations less than 1.0: solid is for F0 (β=0.067), dashed is for F1 (β=0.109).

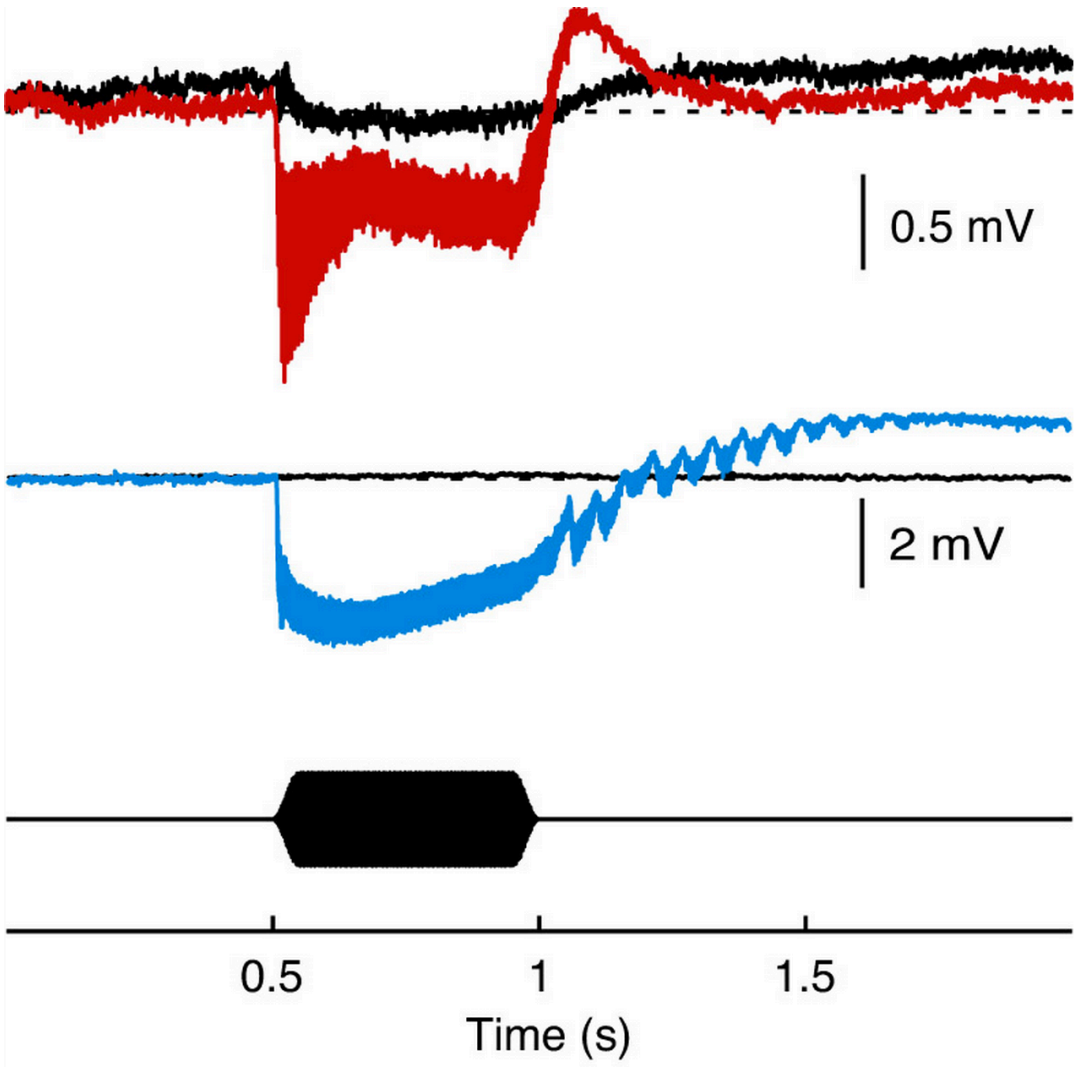


Fig. S2.7. Sustained deflections in Johnston's organ of a female*Drosophila* (red) and the mosquito Aedes aegypti blue; from Cator et al. (Cator et al., 2009). Stimuli were 400 Hz tones at 107 dB in the fruit flies and 115 dB in the mosquito. Note the greater voltage scale for the mosquito.

CHAPTER 3

ASSOCIATIVE LEARNING IN THE DENGUE VECTOR MOSQUITO, *AEDES AEGYPTI*: AVOIDANCE OF PREVIOUSLY ATTRACTIVE ODOR OR SURFACE COLOR THAT IS PAIRED WITH AN AVERSIVE STIMULUS

Abstract

Associative learning has been shown in a variety of insects, including the mosquitoes *Culex quinquefasciatus* and *Anopheles gambiae*. This study demonstrates associative learning for the first time in *Aedes aegypti*, an important vector of dengue, yellow fever and chikungunya viruses. This species prefers to rest on dark surfaces and is attracted to the odor of 1-octen-3-ol. After training in which a dark surface alone or a dark surface with odor was paired with electric shock, mosquitoes avoided the previously attractive area. The association was stronger when odor was included in training, was retained for at least 60 min but not for 24 h, and was equal for males and females. These results demonstrate the utility of a bulk-training paradigm for mosquitoes similar to that used with *Drosophila melanogaster*.

*Menda, G. *et al.* Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. *J. Exp. Biol.* 216, 218–223 (2013)

INTRODUCTION

It has long been known that insects are capable of associative learning, with most work concentrating on bees (Menzel, 1999; Giurfa, 2007; Giurfa and Sandoz, 2012) and flies (Davis, 2005; Keene and Waddell, 2007). While other insects have also been studied in depth, including cockroaches, moths, wasps, and solitary and social bees (Dukas, 2008), there have been few rigorous studies of learning in mosquitoes. This is surprising given their significant impact on human and animal health and aspects of their life cycle and mode of feeding that may be mediated by learning (Clements, 1992).

Aedes aegypti is a tropical mosquito that feeds preferentially on humans (Harrington et al., 2001) and is a vector for dengue, yellow fever and chikungunya viruses (Gubler, 1998; World Health Organization, 2002; Ligon, 2006). Nearly 40% of the world population may be exposed to dengue, with over 100 million infected and 22,000 fatalities annually (World Health Organization, 1997). There is no vaccine or treatment for dengue infection, so vector control is currently the only means of fighting the disease (Swaminathan and Khanna, 2009; Webster et al., 2009). However, the usual methods of control, reducing breeding sites and applying insecticides, have been of only limited success (World Health Organization, 1997; Gubler, 1998; Ooi et al., 2006). Understanding the role of learning in mosquito behavior could explain choice of breeding sites and hosts, which could in turn give rise to new control methods. Several researchers have suggested that learning could be involved in preference for nectar sources (Jhumur et al., 2006), host species (Hii et al., 1991; Mwandawiro et al., 2000) or even

individuals of a host species (McCall and Kelly, 2002), choice of oviposition sites (Kaur et al., 2003), and home range (Charlwood et al., 1988; McCall et al., 2001).

The existing body of research into mosquito learning is small and contradictory, with some finding no evidence of learning in *Ae. Aegypti* (Alonso et al., 2003) and others claiming learning in various species but with flawed methods (reviewed by Alonso and Schuck-Paim, 2006). Of the few studies with clear evidence of learning, two studies show associative learning with appetitive stimuli in *Culex* (Tomberlin et al., 2006; Sanford and Tomberlin, 2011) and one study shows associative learning with appetitive stimuli in *Anopheles* (Chilaka et al., 2012). Our current work examines associative learning with aversive stimuli in *Ae. aegypti*, using bulk training methods similar to those established with *Drosophila melanogaster* (Quinn et al., 1974).

There are many forms of associative learning. In most of them, an animal learns to associate a neutral stimulus (the conditional stimulus) with a positive or negative stimulus (the unconditional stimulus). After trials in which the two stimuli are paired, the subject responds to the conditional stimulus alone as if the unconditional stimulus were present. We used an inhibitory avoidance test to determine whether mosquitoes could learn to avoid innately attractive stimuli. Inhibitory avoidance learning is often used to test the effect of drugs and neuromodulators on learning in rodents (e.g. McIntyre et al., 2002). In one version of the task, a rat is placed in a brightly lit area (aversive) and permitted to enter a dark area (innately preferred). However, the floor of the dark area delivers a shock. Increased latency or reduced probability of

entering the dark area is taken as evidence of learning. Inhibitory avoidance learning differs from classical conditioning in two important respects. First, the conditional stimulus must be attractive rather than neutral. Second, it is not possible to have a control in which conditional and unconditional stimuli are unpaired during training. Instead, control groups go through the training procedure without exposure to shock.

We tested two attractive stimuli, a color and an odor. Both sexes of *Ae. aegypti* prefer to rest on dark-colored surfaces (Gilbert and Gouck, 1957) and are attracted to the odor of 1-octen-3-ol (Takken and Kline, 1989; Grant and Dickens, 2011). These are the two conditional stimuli, while the aversive unconditional stimulus is an electric shock delivered through the dark surface. If associative learning occurs, the proportion of mosquitoes resting on the dark surface should decrease after training.

MATERIALS AND METHODS

Mosquitoes

For this study, *Aedes aegypti* L. came from a lab colony established from eggs collected in Tapachula, Mexico (14°54'N, 92°15'W) in 2006 and supplemented with field-collected eggs from the same region in 2008 and 2009. Mosquitoes were kept in an environmental chamber simulating natural conditions, with a 14 h:10 h light:dark cycle and 2 h of dawn and twilight, at 75±7% relative humidity and at 22–30°C fluctuating temperature. Eggs were vacuum-hatched in water to obtain simultaneous cohorts. Larvae were fed 1:1 lactalbumin and brewer's yeast. Male and female pupae were transferred to separate 2-liter containers with mesh lids and offered a 20% sucrose solution upon eclosion. No more than 45 pupae were placed in a container. Containers of adult mosquitoes were kept in the environmental chamber until the day of an experiment.

Experimental chamber

Experiments took place in a 46-cm long, 9.5-cm inner-diameter transparent Plexiglas^{TM} cylinder closed at both ends (Fig 3.1.). One end cap was white plastic, while the other end cap was a darker printed-circuit board (PCB) through which an electric shock could be applied. The PCB had interleaved hot and neutral contacts separated by 1 mm. The outside of the chamber was mostly covered with white paper; to facilitate counting of mosquitoes, 2 cm by the walls at each end and a strip at the top were left uncovered. Six 2.5 mm-diameter holes were drilled in the cylinder wall, 5 mm from the PCB end:

two for delivery of odor, two for removal of odor, and two for pressure release. Four similar holes provided comparable air circulation at the other end of the cylinder. During experiments, a desk lamp illuminated the chamber, with intensity ranging from 700 lux at the end caps to 1270 lux in the center of the chamber (approximately the brightness of an overcast day). We defined two areas of the chamber, a dark area consisting of the PCB and adjacent 2 cm of cylinder, and a light area consisting of the white end cap and the remaining 44 cm of cylinder (Fig 3.1.). The dark area was ~9% of the total interior surface of the chamber.

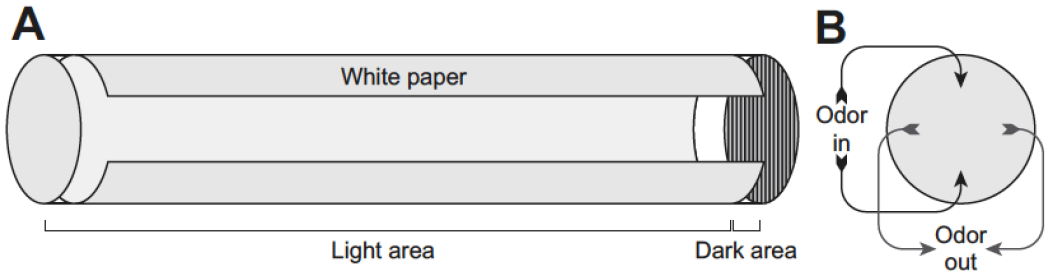


Fig 3.1 Experimental chamber. (A) Mosquitoes were released in a 46 cm long, 9.5 cm inner-diameter cylinder. One end cap was a dark printed-circuit board (PCB) through which electric shock could be applied; the other end was white. To assess the preference of mosquitoes, we defined a dark area consisting of the PCB and adjacent 2 cm of cylinder, comprising 9% of the total interior surface area. (Darkness refers to surface color rather than illumination, which was similar throughout the chamber.) (B) For odor experiments, odor was pumped in through two holes at the dark end and simultaneously pumped out through two orthogonally placed holes.

Stimuli

The unconditional stimulus was a 100 mA, 140 V AC shock applied through the PCB at the dark end of the experimental chamber for the 60 s of a training trial. This intensity caused most mosquitoes to leave the PCB without any evident harm. We tested two conditional stimuli: (1) the attractively dark-colored PCB wall and (2) the odor of 1-octen-3-ol (98%, Acros lot A0272468). The color was always present, while the odorant was delivered only during the 60 s of a trial. Since the dark PCB was always present, our two conditional stimuli were 'dark surface color alone' and 'dark surface color with odor'. We henceforth refer to these as 'color' and 'odor/color', respectively.

To deliver odor to the dark area of the chamber, air was pumped through a 20 ml vial containing filter paper impregnated with 1.5 ml of odorant and then into the experimental chamber. The pump (Micro Air Pump, part 3A120INSN, 475 cm^3 min^{-1}) moved air into the chamber through two opposite ports near the PCB, while a vacuum pump (Metal Bellows model MB-41) simultaneously removed air through two orthogonal ports, and two additional holes in the chamber kept pressure equalized. To avoid odorant buildup, we cleaned the vial before each test. After each experiment, we cleaned the chamber with ethanol to remove any residues and left it to dry overnight.

Procedure

Training and testing procedures are shown schematically in Fig 3. 2. Before an experiment, a cohort of 35–40 mosquitoes of the same sex and age (2–10 days post-eclosion) was anesthetized by chilling for 30 s at 5°C and then transferred to the experimental chamber described above. They were given 5 min to acclimate to the chamber before experiments began.

We determined the dark-area preference of mosquitoes as follows. The chamber was gently shaken (to make mosquitoes fly), rotated (to avoid side bias), shaken again and then placed on a table. This took ~5 s, after which mosquitoes were given 35 s to settle. We then counted mosquitoes in the dark and light areas of the chamber and calculated the proportion resting in the dark area. We did this at the outset of each experiment, to establish a baseline, and at intervals after training to test learning and retention. In odor tests, the odor was present in the dark-surface area during this time.

There were four types of trials, each lasting 60 s: color + shock, color control, odor/color + shock, and odor/color control. Each training trial with shock was as follows. The chamber was shaken, turned and shaken as above. Electrical current to the PCB was turned on for 60 s. In odor trials, the odor was supplied for 60 s along with the current. Control trials were the same except that no current was supplied to the PCB. The interval between trials was 60 s, so a trial occurred every 120 s.

Each cohort was used in only one experiment with one type of training or

control trial. An experiment consisted of an initial baseline test of dark-area preference, nine training or control trials, a 10 min rest period, a test of dark-area preference, a 60 min rest period, another test of dark-area preference, and five more training trials followed 10 min later by a final test of dark-area preference. This gave us four measures of dark-area preference: baseline, 10 min after first training, 60 min after first training, and 10 min after second training.

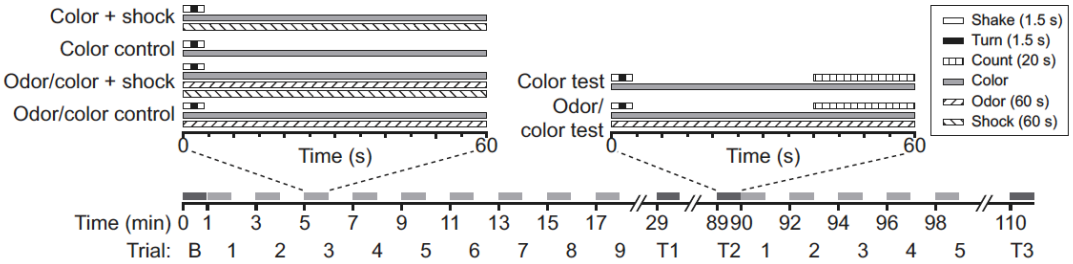


Fig. 3.2 Procedure. The time line shows the pattern of test trials (dark bars) and training trials (light bars). On the Trial axis, B denotes the baseline preference test before training, T1 denotes a preference test 10 min after first training, T2 denotes a test 60 min after first training, and T3 denotes a test 10 min after a second training, while numbers represent training trials. The content of each trial or test depended on the type of experiment, as shown in the expanded time lines and described in the text. Color refers to trials or tests in which only the dark surface color was present; odor/color refers to trials or tests in which the odor was present along with the dark surface color.

To test retention beyond 60 min, we ran a separate set of experiments (not shown in Fig 3. 2) with 12 cohorts trained with odor/color + shock and 12 odor/color control cohorts; all were female. Baseline preference was tested, followed by nine training or control trials, followed by a preference test after another 24 h in the chamber.

Because mosquitoes remained in the chamber between training and test trials, a further control was needed. It is possible that shocked mosquitoes emit a substance that repels other mosquitoes. To test this, we placed a cohort in the chamber, shook and rotated them, and counted the number in the dark area. Those mosquitoes then experienced nine trials with shock, shaking and rotation on the same schedule as the color + shock training trials. We then removed those mosquitoes, immediately replaced them with a naive cohort, shook and rotated the chamber, and counted the number in the dark area. If the shocked mosquitoes leave an alarm substance, then naïve mosquitoes should avoid the dark area.

Statistics

Each cohort of 35–40 mosquitoes was measured several times in the course of an experiment: once at the outset, twice after the initial training, and once after a second training. Each experimental treatment used 12 cohorts. To assess learning and retention, we tested several predetermined comparisons, each of them separately for males and females. Predetermined comparisons of baseline values were done with ANOVA. For predetermined comparisons between tests and baselines, between control and experimental groups, and between stimulus types,

we used paired *t*-tests with Bonferroni corrections for multiple comparisons (four for any comparison with baseline, three for tests among post-baseline experimental and control values). All *t*-tests had 22 degrees of freedom, with 12 cohorts per sample. Finally, testing for differences between males and females was done with a multilevel model. Fixed effects in this model were sex (male or female), conditional stimulus (color or odor/color), experiment (shock or control), treatment (baseline, 10 min after first training, 60 min after first training, 10 min after second training), and all interactions. Cohort was set as a random effect (12 cohorts of each sex were run through each experiment). All tests were done with JMP software (SAS Institute, Inc., Cary, NC, USA). All *P*-values reported below are corrected for multiple comparisons as necessary.

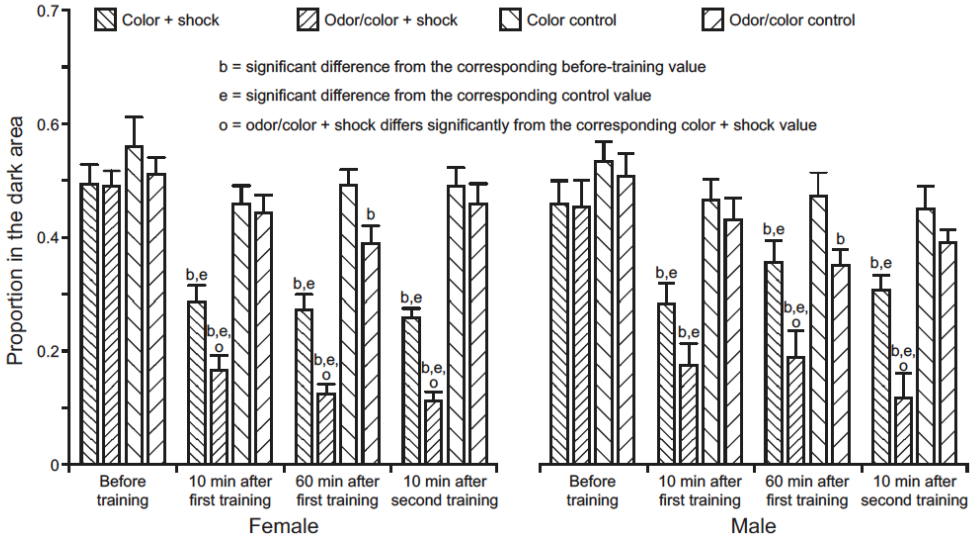


Fig. 3.3. Results. Bars show the proportion of mosquitoes counted in the dark area of the chamber (mean ± s.e.m.); decrease from the initial value indicates aversive learning. Labels below bars correspond to tests described in Fig. 2. All experimental test values differed significantly from baseline; control values differed significantly from baseline in only two cases. All experimental values after the baseline test differed significantly from their corresponding controls. After training, values for odor/color + shock were significantly lower than for color + shock except in one case. There were no statistically significant differences between males and females. All *t*- and *P*-values are given in the text.

RESULTS

Baseline measurements for both sexes confirmed that the dark area was attractive on its own, with or without the presence of the odor. The dark area was only 9% of the internal surface of the chamber, yet over 50% of mosquitoes were found in that area before training (Fig 3. 3). There were no statistically significant differences in baseline preference among cohorts used in the different experiments (ANOVA: female P=0.11, $F_{3,22}$=2.62; male P=0.23, $F_{3,22}$=1.48). For each sex, five replications of the control experiment for alarm substances showed no significant difference between the distribution of mosquitoes in a clean chamber and that of naive mosquitoes placed in a chamber after its prior occupants received nine electric shocks (ANOVA: female pre- shock 43±2% in dark area, naive 43±3% in dark area, $F_{1,8}$=0.07, P=0.80; male pre-shock 46±2% in dark area, naive 51±4% in dark area, $F_{1,8}$=0.90, P=0.37). Thus, shocked mosquitoes do not alter their surroundings in any way that deters other mosquitoes, so changes in mosquito distribution after training indicate learning rather than aversion to an alarm substance.

The inhibitory avoidance paradigm makes three straightforward predictions. (1) If mosquitoes learn to associate the conditional stimulus (color or odor/color) with the aversive stimulus (shock), then their preference for the dark or odor/dark area should decrease after training. (2) Control experiments, in which training trials lack a shock, should show no decline from the baseline preference for the dark area. (3) Preference of trained mosquitoes for the dark area should be less than that of control mosquitoes.

Based on these predictions, we judged learning by the extent to which preference for the dark area decreased from baseline. We judged retention by the extent to which that preference remained depressed at intervals after training.

The first prediction was met, since the proportion of mosquitoes resting in the dark area 10 min after first training was less than baseline for both sexes and stimuli ($P<0.0001$ in all tests; female color, $t=5.52$; female odor/color, $t=8.67$; male color, $t=4.65$; male odor/color, $t=7.45$). The second prediction was met, since the proportion of control mosquitoes in the dark area did not differ significantly from the baseline after the first training for either sex or stimulus except in one case (female color $P=0.03$, $t=2.69$; female odor/color $P=0.28$, $t=1.81$; male color $P=0.29$, $t=1.80$; male odor/color $P=0.16$, $t=2.07$). The third prediction was also met, since the proportion of trained mosquitoes preferring the dark area after training was less than that of the control mosquitoes for both sexes and stimuli (female color $P=0.0012$, $t=3.57$; female odor/color $P<0.0001$, $t=5.76$; male color $P=0.0006$, $t=3.77$; male odor/color $P<0.0001$, $t=5.31$). Results after the second training period were comparable.

The association was retained for at least 60 min after training. Preference for the dark area remained below baseline for both sexes and stimuli (female color $P<0.0001$, $t=5.89$; female odor/color $P<0.0001$, $t=5.52$; male color $P=0.025$, $t=2.75$; male odor/color $P<0.0001$, $t=7.07$) and was less than that of control mosquitoes (female color $P<0.0001$, $t=4.54$; female odor/color $P<0.0001$, $t=5.46$; male color $P=0.045$, $t=2.45$; male odor/color $P=0.003$,

t=3.33). However, dark-area preference of the control mosquitoes declined slightly and differed significantly from baseline in two cases (female color *P*=0.29, *t*=1.81; female odor/color *P*=0.005, *t*=3.29; male color *P*=0.44, *t*=1.61; male odor/color *P*=0.0002, *t*=4.18). In the 24-h retention test (all females, odor/color only), there was no difference between trained and control mosquitoes (*P*=1.0, *t*=0.031) and neither group differed significantly from its baseline (trained *P*=0.10, *t*=2.08; control *P*=0.25, *t*=1.60). Association between the conditional stimulus and shock was stronger with the odor/color combination than with color alone. The dark-area preference was significantly lower after odor/color training than after color training in females (T1 *P*=0.04, *t*=2.51; T2 *P*=0.007, *t*=3.06; T3 *P*=0.008, *t*=3.02) and males except at 10 min after first training (T1 *P*=0.07, *t*=2.27; T2 *P*=0.002, *t*=3.44; T3 *P*=0.0003, *t*=3.94).

Although dark-area preference was sometimes lower for trained females than trained males, sex was never statistically significant either as a main effect, or in any interaction terms, or in any *post- hoc* comparisons in our mixed multilevel model.

DISCUSSION

In summary, our data show that *Ae. aegypti* learned to avoid previously attractive stimuli paired with a shock in a bulk-training paradigm. The association was retained for at least 60 min but not for 24 h, was stronger for color with odor than for color alone, and was equal for males and females. Reversal of preference after training has also been shown in honeybees, which learned aversion to attractant pheromones and floral odorants, but has been investigated in few other insects (Roussel et al., 2012).

Our findings are at odds with the only other study that has tested associative learning in *Ae. aegypti* (Alonso et al., 2003). That series of experiments paired aversive (shock or vibration) or positive (blood feeding or human breath) unconditional stimuli with neutral conditional stimuli (odors or visual patterns) and found no evidence of associative learning with any pairing. While their procedures differed from ours in many respects, including a relatively short training period, there are no obvious flaws in their design. Alonso et al. speculate that *Ae. aegypti* may not need to learn in nature but also acknowledge that their mosquitoes (from a colony founded in the 1950s) may show the effect of many generations of laboratory rearing (Alonso et al., 2003). In contrast, our mosquitoes came from a colony collected from the wild in 2006 and refreshed in 2009. We suspect that this accounts for the discrepant results. Recent work in *D. melanogaster* found a genetic polymorphism affecting learning (Mery et al., 2007) and metabolic costs to memory (Mery and Kawecki, 2005). This is probably also true of mosquitoes, making it likely that learning ability could be reduced over many generations of lab

rearing, due to genetic drift and relaxed selection.

Three recent studies have successfully shown associative learning in other mosquito species. Two used appetitive stimuli with *Culex quinquefasciatus* (Tomberlin et al., 2006; Sanford and Tomberlin, 2011). In both cases, individual mosquitoes learned to associate previously neutral odors with food reward (sugar or blood). A bulk- training study showed that female *Anopheles gambiae* could associate visual or olfactory stimuli with desirable and undesirable feeders or normal and unpalatable blood (Chilaka et al., 2012). Learning was rapid, with mosquitoes reaching 100% accuracy on the fourth trial with visual stimuli and palatable *versus* unpalatable blood.

The initial bulk training studies in *Drosophila* (Quinn et al., 1974) were criticized for not directly measuring the learning of individuals, and the matter has been discussed at length (e.g. Holliday and Hirsch, 1986; McGuire, 1986; Tully, 1986). However, it is clear that group learning reflects individual learning even when individual learning is not directly measured. Indeed, if no individuals learn, there can be no change in the behavior of a trained group. Individual training (as in the *Culex* work) and bulk training (as in our work and the *Anopheles* work) have inevitable trade-offs. Individual training offers precise control over conditions such as age, appetitive state, and the timing of conditional and unconditional stimuli, and may reveal nuances of behavior and individual variation among subjects.

However, it is time-consuming, requires careful handling of delicate insects and could be difficult to scale up for use with large numbers. Bulk training accepts some variability in the treatment experienced by each subject and uncertainty

about variation among subjects in exchange for ease of implementation. There is no need to handle mosquitoes individually; response measurement is simple, requiring no judgments about the quality of a behavior; and large amounts of data can be rapidly collected. In our method, additionally, the appetitive state of the mosquitoes is not critical and the unconditional stimulus is salient to both sexes.

Bulk training was clearly effective in our tests. However, some aspects of the method may have reduced its sensitivity. (1) Not all mosquitoes in a cohort got the aversive stimulus in each trial. At the outset, about 50% were on the PCB and received a shock; as the number on the PCB declined, the number receiving the shock also declined. Thus, over nine training trials, mosquitoes experienced fewer than nine pairings of conditional and unconditional stimuli.

(2) By necessity, the dark PCB was always present, so mosquitoes in color + shock and odor/color + shock experiments could land on it between trials without receiving a shock. These experiences amounted to extinction training, which may have reduced during training and short-term retention tests, extinction probably occurred in the 24-h retention test, since mosquitoes went through a circadian cycle and redistributed themselves. (3) If shaking is aversive, all mosquitoes got an aversive stimulus on each trial and may have associated it with whatever surface they were on. Since many mosquitoes started out on the dark surface, this may account for the small, generally non-significant, decline in dark-area preference over time in control experiments.

We found retention for 60 min but not 24 h after aversive training. This is in contrast to 24-h retention after appetitive conditioning in *Culex* (Sanford and Tomberlin, 2011) and 72-h retention after appetitive conditioning in *Anopheles* (Chilaka et al., 2012). There are four possible explanations. First, it has long been known that learning is retained longer after widely separated (spaced) than closely separated (massed) training trials. For example, honeybees trained with a 180-s intertrial interval showed significantly greater retention after 24–72 h than those trained with a 30-s intertrial interval (Menzel et al., 2001). Trial spacing may explain the 72-h retention in *Anopheles* [300-s interval (Chilaka et al., 2012)] but does not easily explain the difference between retention in *Aedes* (<24 h retention after 60-s interval; current study) and *Culex* [24-h retention after 30-s interval (Sanford and Tomberlin, 2011)]. The *Culex* and *Aedes* studies both used massed trials but differed in retention at 24 h. Second, appetitive and aversive learning may differ. One study of *D. melanogaster* larvae found that, with identical training paradigms, appetitive odor association long outlasted aversive odor association (Honjo and Furukubo-Tokunaga, 2009). This may also be true of mosquitoes, although we are cautious about drawing that conclusion from three different species and training paradigms. Third, learned aversion to an innately preferred stimulus may be weaker, and more readily lost, than an aversive association attached to an otherwise neutral stimulus. Finally, we cannot exclude the possibility that lack of retention in our test was due to *de facto* extinction training during the 24-h period, as described above.

Although the baseline attractiveness of the dark surface with odor did not

differ from that of the dark surface alone, odor clearly affected learning. Mosquitoes learned to associate the dark surface with shock, but the association was stronger when odor was also present. Our experiments were not intended to address questions of blocking, overshadowing, configural *versus* elemental stimuli, or stimulus generalization that arise when conditional stimuli are used in combination (Pearce, 1987). Future work might address some of these issues by training with odor and color together and testing with color alone (to test overshadowing) or by training with odor in a chamber with dark PCBs at both ends (to test the strength of odor as a conditional stimulus on its own).

We found no sex difference in learning, nor have others who have looked for it in mosquitoes (Sanford and Tomberlin, 2011), fruit fly larvae (Neuser et al., 2005) or honeybees (Bitterman et al., 1983). However, one study found that female honeybees learn better than males in tasks specifically related to foraging behavior (Shafir et al., 2005). Given that female and male mosquitoes have very different feeding behaviors and risks to balance in foraging and reproduction, it is likely that sex-specific learning assays could be found for them as well.

Naturalistic studies of mosquito learning have been problematic (reviewed by Alonso and Schuck-Paim, 2006), but there are two that show clear evidence of learning. Mwandawiro et al. found that three species of *Culex* preferred hosts to which they had been previously exposed; they found no such preference in the species of *Aedes* that they tested (Mwandawiro et al., 2000). Similarly, female *Culex* raised as larvae in water containing skatole

preferred as adults to oviposit in skatole-containing water over plain water or water containing *p*-cresol, despite the fact that skatole is normally repellant and *p*-cresol normally attractive (McCall and Eaton, 2001). Although these two studies showed learning and successfully excluded genetic predisposition (daughters did not show the same preference as their mothers), the type of learning underlying these behaviors was not established. Learned aversion to host odors may explain heterogeneous distribution of disease vectors among host individuals and may provide an avenue for targeted control methods (McCall and Kelly, 2002). Learned aversion to a host odor has been demonstrated in the laboratory with bloodsucking bugs (Vinauger et al., 2011) but has not been reported with mosquitoes.

Finding that *Ae. aegypti* is capable of associative learning is not surprising, given that it has been demonstrated in other insects of similar size and behavioral complexity. Indeed, the opposite finding, inability to learn, would require special explanation. A more practical question is whether mosquitoes use this ability in nature. Given their complex life history, there is likely to be an advantage to learning but this remains to be shown in free-ranging mosquito populations. Further lab studies could direct the design of well- controlled field experiments or naturalistic lab experiments by circumscribing the parameters within which mosquitoes learn. For example, lab work might show what stimuli are adequate as conditional or unconditional stimuli, reveal the minimum number of training trials required, or show the effects of age, sex, circadian state or appetitive state.

Bulk training methods such as ours and the recent work on *Anopheles* (Chilaka et al., 2012) provide a framework for large-scale studies of mosquito learning.

ACKNOWLEDGEMENTS

We thank Sylvie Pitcher, Melissa Orteza, and other members of the Harrington laboratory for mosquito rearing and maintenance, Gary Oltz for assistance with construction of the test apparatus, Haim Bar for additional statistical consulting, and three anonymous reviewers for useful comments.

FUNDING

This research was supported by the National Institutes of Health [5R01DC103-37 to R.R.H.] and a Hatch award [2010-11-184 to L.C.H.]. Deposited in PMC for release after 12 months.

REFERENCES

Alonso, W. J. and Schuck-Paim, C. (2006). The 'ghosts' that pester studies on learning in mosquitoes: guidelines to chase them off. *Med. Vet. Entomol.* **20**, 157- 165.

Alonso, W. J., Wyatt, T. D. and Kelly, D. W. (2003). Are vectors able to learn about their hosts? A case study with *Aedes aegypti* mosquitoes. *Mem. Inst. Oswaldo Cruz* **98**, 665-672.

Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107- 119.

Charlwood, J. D., Graves, P. M. and Marshall, T. F. (1988). Evidence for a 'memorized' home range in *Anopheles farauti* females from Papua New Guinea. *Med. Vet. Entomol.* **2**, 101-108.

Chilaka, N., Perkins, E. and Tripet, F. (2012). Visual and olfactory associative learning in the malaria vector *Anopheles gambiae sensu stricto*. *Malar. J.* **11**, 27.

Clements, A. (1992). *The Biology of Mosquitoes: Development, Nutrition and Reproduction*. London, UK: Chapman and Hall.

Davis, R. L. (2005). Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. *Annu. Rev. Neurosci.* **28**, 275-302.

Dukas, R. (2008). Evolutionary biology of insect learning. *Annu. Rev. Entomol.* **53**, 145-160.

Gilbert, I. and Gouck, H. (1957). Influence of surface color on mosquito landing rates. *J. Econ. Entomol.* **50**, 678-680.

Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **193**, 801-824.

Giurfa, M. and Sandoz, J. C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* **19**, 54-66.

Grant, A. J. and Dickens, J. C. (2011). Functional characterization of the octenol receptor neuron on the maxillary palps of the yellow fever mosquito, *Aedes aegypti. PLoS ONE* **6**, e21785.

Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.* **11**, 480-496.

Harrington, L. C., Edman, J. D. and Scott, T. W. (2001). Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? *J. Med. Entomol.* **38**, 411-422.

Hii, J. L., Chew, M., Sang, V. Y., Munstermann, L. E., Tan, S. G., Panyim, S. and Yasothornsrikul, S. (1991). Population genetic analysis of host seeking and resting behaviors in the malaria vector, *Anopheles balabacensis* (Diptera: Culicidae). *J. Med. Entomol.* **28**, 675-684.

Holliday, M. and Hirsch, J. (1986). A comment on the evidence for learning in Diptera. *Behav. Genet.* **16**, 439-447.

Honjo, K. and Furukubo-Tokunaga, K. (2009). Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in *Drosophila* larvae. *J. Neurosci.* **29**, 852-862.

Jhumur, U. S., Dötterl, S. and Jürgens, A. (2006). Naive and conditioned responses of *Culex pipiens pipiens* biotype molestus (Diptera: Culicidae) to flower odors. *J. Med. Entomol.* **43**, 1164-1170.

Kaur, J. S., Lai, Y. L. and Giger, A. D. (2003). Learning and memory in the mosquito *Aedes aegypti* shown by conditioning against oviposition deterrence. *Med. Vet. Entomol.* **17**, 457-460.

Keene, A. C. and Waddell, S. (2007). *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci.* **8**, 341-354.

Ligon, B. L. (2006). Reemergence of an unusual disease: the chikungunya epidemic. *Semin. Pediatr. Infect. Dis.* **17**, 99-104.

McCall, P. J. and Eaton, G. (2001). Olfactory memory in the mosquito *Culex quinquefasciatus*. *Med. Vet. Entomol.* **15**, 197-203.

McCall, P. J. and Kelly, D. W. (2002). Learning and memory in disease vectors. *Trends Parasitol.* **18**, 429-433.

McCall, P. J., Mosha, F. W., Njunwa, K. J. and Sherlock, K. (2001). Evidence for memorized site-fidelity in *Anopheles arabiensis*. *Trans. R. Soc. Trop. Med. Hyg.* **95**, 587-590.

McGuire, T. R. (1986). Further evidence for learning in Diptera: a reply to Holliday and Hirsch. *Behav. Genet.* **16**, 457-473.

McIntyre, C. K., Hatfield, T. and McGaugh, J. L. (2002). Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. *Eur. J. Neurosci.* **16**, 1223-1226.

Menzel, R. (1999). Memory dynamics in the honeybee. *J. Comp. Physiol. A* **185**, 323- 340.

Menzel, R., Manz, G., Menzel, R. and Greggers, U. (2001). Massed and spaced learning in honeybees: the role of CS, US, the intertrial interval, and the test interval. *Learn. Mem.* **8**, 198-208.

Mery, F. and Kawecki, T. J. (2005). A cost of long-term memory in *Drosophila*. *Science* **308**, 1148.

Mery, F., Belay, A. T., So, A. K., Sokolowski, M. B. and Kawecki, T. J. (2007). Natural polymorphism affecting learning and memory in Drosophila. *Proc. Natl. Acad. Sci. USA* **104**, 13051-13055.

Mwandawiro, C., Boots, M., Tuno, N., Suwonkerd, W., Tsuda, Y. and Takagi, M. (2000). Heterogeneity in the host preference of Japanese

encephalitis vectors in Chiang Mai, northern Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **94**, 238-242.

Neuser, K., Husse, J., Stock, P. and Gerber, B. (2005). Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type and memory span. *Anim. Behav.* **69**, 891-898.

Ooi, E.-E., Goh, K.-T. and Gubler, D. J. (2006). Dengue prevention and 35 years of vector control in Singapore. *Emerg. Infect. Dis.* **12**, 887-893.

Pearce, J. M. (1987). A model for stimulus generalization in Pavlovian conditioning. *Psychol. Rev.* **94**, 61-73.

Quinn, W. G., Harris, W. A. and Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **71**, 708-712.

Roussel, E., Padie, S. and Giurfa, M. (2012). Aversive learning overcomes appetitive innate responding in honeybees. *Anim. Cogn.* **15**, 135-141.

Sanford, M. R. and Tomberlin, J. K. (2011). Conditioning individual mosquitoes to an odor: sex, source, and time. *PLoS ONE* **6**, e24218.

Shafir, S., Menda, G. and Smith, B. H. (2005). Caste-specific differences in risk sensitivity in honeybees, *Apis mellifera. Anim. Behav.* **69**, 859-868.

Swaminathan, S. and Khanna, N. (2009). Dengue: recent advances in biology and current status of translational research. *Curr. Mol. Med.* **9**, 152-173.

Takken, W. and Kline, D. L. (1989). Carbon dioxide and 1-octen-3-ol as mosquito attractants. *J. Am. Mosq. Control Assoc.* **5**, 311-316.

Tomberlin, J. K., Rains, G. C., Allan, S. A., Sanford, M. R. and Lewis, W. J. (2006). Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae). *Naturwissenschaften* **93**, 551-556.

Tully, T. (1986). Measuring learning in individual flies is not necessary to study the effects of single-gene mutations in *Drosophila*: a reply to Holliday and Hirsch. *Behav. Genet.* **16**, 449-455.

Vinauger, C., Buratti, L. and Lazzari, C. R. (2011). Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. II. Aversive learning. *J. Exp. Biol.* **214**, 3039-3045.

Webster, D. P., Farrar, J. and Rowland-Jones, S. (2009). Progress towards a dengue vaccine. *Lancet Infect. Dis.* **9**, 678-687.

World Health Organization (1997). *Vector Surveillance and Control Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control.* Geneva, CH: World Health Organization.

World Health Organization (2002). *Dengue and Dengue Haemorrhagic Fever.* Geneva, CH: World Health Organization.

CHAPTER 4

VISUAL PERCEPTION IN THE BRAIN OF A JUMPING SPIDER.

ABSTRACT

We report neurophysiological recordings from the brain of a jumping spider, Phidippus audax (Salticidae). The data include single-unit recordings in response to artificial and naturalistic visual stimuli. The salticid visual system is unique in that high acuity and motion vision are processed by different pairs of eyes. We found nonlinear interactions between the principal and secondary eyes, which can be inferred from the emergence of spatiotemporal receptive fields. Ecologically relevant images, including prey-like objects such as flies, elicited bursts of excitation from single units. This initial probe into the neurophysiological basis of salticid behavior shows that the remarkable and curious behavior of jumping spiders can be investigated at the level of individual neurons.

- In review as Menda at el. In Current Biology.

RESULTS AND DISCUSSION

Jumping spiders (Salticidae) are renowned for a behavioral repertoire that can seem more vertebrate, or even mammalian, than spider-like in character [1] [2] [3]. This is made possible by a unique visual system that supports their stalking hunting style and elaborate mating rituals in which the bizarrely marked and colored appendages of male salticids highlight their song-and-dance displays [4] [2] [5]. Salticids perform these tasks with information from four pairs of functionally specialized eyes, providing a near 360-degree field of view (Figure 4.1) and forward-looking spatial resolution surpassing that of all insects and even some mammals [1], processed by a brain roughly the size of a poppy seed. Salticid behavior, evolution, and ecology are well documented [6] [7] [8], but attempts to study the neurophysiological basis of their behavior had been thwarted by the pressurized nature of their internal body fluids. As in all spiders, the segment that houses the brain (the prosoma, akin to the cephalothorax of other arthropods, like lobsters) is maintained under pressure, making typical physiological techniques infeasible and restricting all previous neural work in salticids to a few recordings from the eyes [9] [10]. Having overcome this challenge (see Supplemental Methods §I-II), we now commence the neurophysiological investigation of jumping spider visual behavior. An extracellular glass-insulated tungsten electrode was inserted through a small hole in the prosoma and into a brain region just posterior to the central bodies (Figure 4.1A)—an area believed to be important for higher-order visual processing [11] [12] [13]. Since this study breaks new ground in terms of physiological techniques, the methods are discussed in detail in the

Supplemental Methods §I-II. Recordings were remarkably stable, often lasting several hours. Sixty-six sites across 34 animals yielded 131.4 hours of total recording time and 20 hours of data selected and analyzed (see Supplemental Methods §VI). Tungsten electrodes necessarily yield extracellular recordings with multiple spiking units (Figure 4.1B). However, conventional spike-sorting techniques [14] allowed us to isolate single units based on waveform amplitude and shape (Figure 4.1B-D). In order to meet statistically imposed benchmarks in the spike sorting process, our data contained thousands of spikes, which were sorted into single units and analyzed offline. As the first investigation of neural processing in the salticid brain, we employed a range of stimuli to explore potential neural correlates of a range of behaviors that make these animals so unique. Each of three stimulus protocols aims to alternatively explore the neural bases of: (1) predatory reactions to moving targets; (2) discrimination between ecologically relevant objects; (3) relationships between different sets of eyes. Further, to conform to established methods [15], we also characterized cells using traditional stimuli (Supplemental Methods §VII-VIII; Figure S4.2).

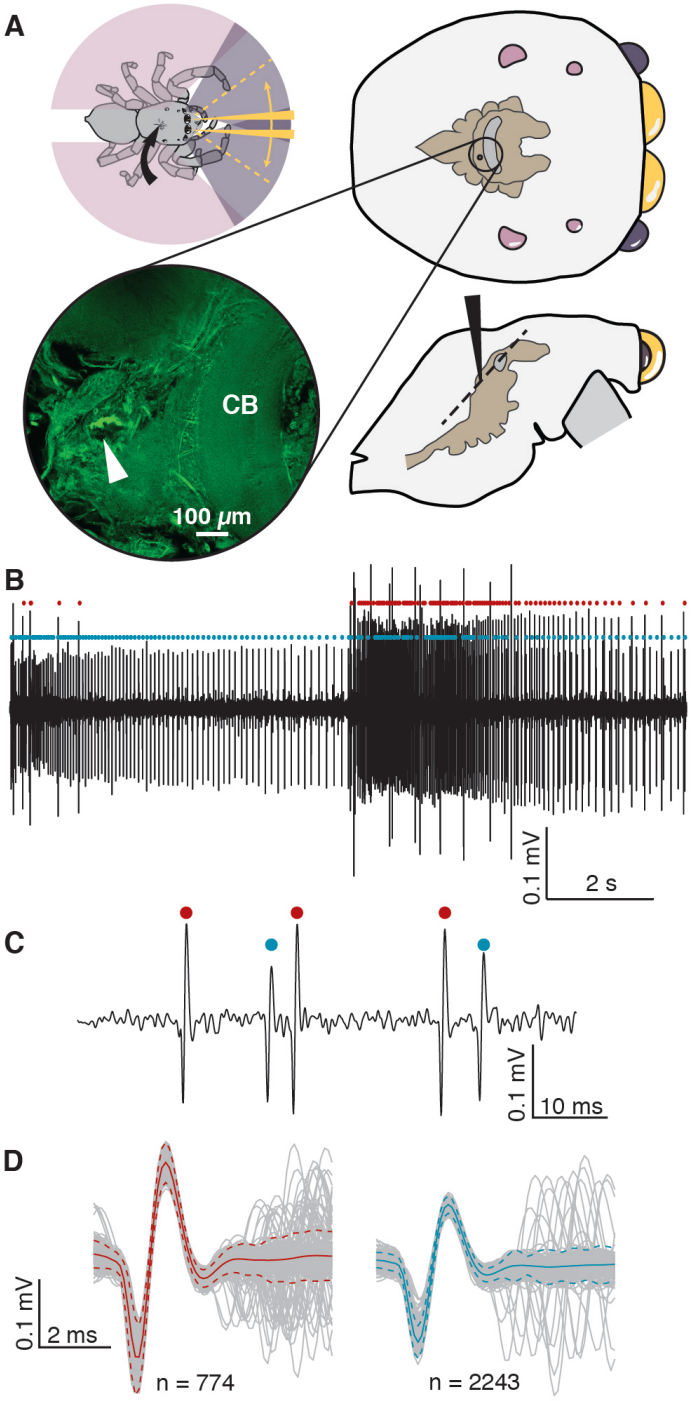

A
CB
100 μm
B
0.1 mV
2 s
C
0.1 mV
10 ms
D
0.1 mV
2 ms
n = 774
n = 2243

FIGURE 4.1.

Recording site and sample recordings.

(A, top left) Approximate fields of view: principal anterior median eyes (yellow) with limits of the movable range indicated by dashed yellow lines, secondary anterior lateral (dark purple), and secondary posterior lateral eyes (light purple). Overlapped fields are represented by more saturated hues. Black arrow shows electrode insertion point. Redrawn from Hill [31] [13]. (A, right) Dorsal (top) and lateral (bottom) view of the prosoma showing the central nervous system (CNS) and eye arrangement; eye colors as indicated previously. Grey in CNS notes the location of the central bodies (CB), a region readily identifiable inhistology and used as a landmark in this study [12]. Black circle in dorsal view and black arrow in lateral view show approximate electrode location and orientation. (A, bottom left) Confocal image verifying electrode location (white arrow) just posterior to the central bodies (CB), evidenced by increased fluorescence surrounding damage
from the electrode. Optical slice depicts area circled in (A, top right) and along plane shown by dashed line in (A, bottom right). (B) Trace from an extracellular recording. In this example two units were identified using a spike sorting algorithm [14] and are marked by red and blue circles. (C) Time-Expanded trace from the same recording session as in (B). (D) Overlay of spikes identified by the spike-sorting algorithm. Colors correspond to spikes shown in parts B and C. Solid line represents the mean; dotted lines are 2 standard deviations from the mean.

1. Prey-sized Moving Targets

Jumping spiders show consistent predatory behavioral responses towards fly lures under laboratory conditions—tracking and pouncing on such targets. Such lures are successful even when they are relatively simple (typically consisting of a dead housefly fixed to a thread or fishing line and moved about in a fly-like manner). While the movements of these lures are only approximations of those of actual prey, we were encouraged to deploy a video version of this stimulus in our experiments because of the behavioral reliability with which salticids respond. At the neural level, our decision to use prey-like stimuli (instead of exclusively exposing spiders to wide-field stimuli such as gratings and lines) is supported by work in other visual systems which has found single neurons that show preferences for small moving targets [16] [17] traits that seem particularly important for predators such as salticids. Responses of single units to our moving prey-like stimulus were extremely robust, with firing patterns showing remarkable trial-to-trial consistency even across 22 repetitions encompassing 24 minutes of experimental recording time (Figure 4.2; video S4.1 and video S4.2).

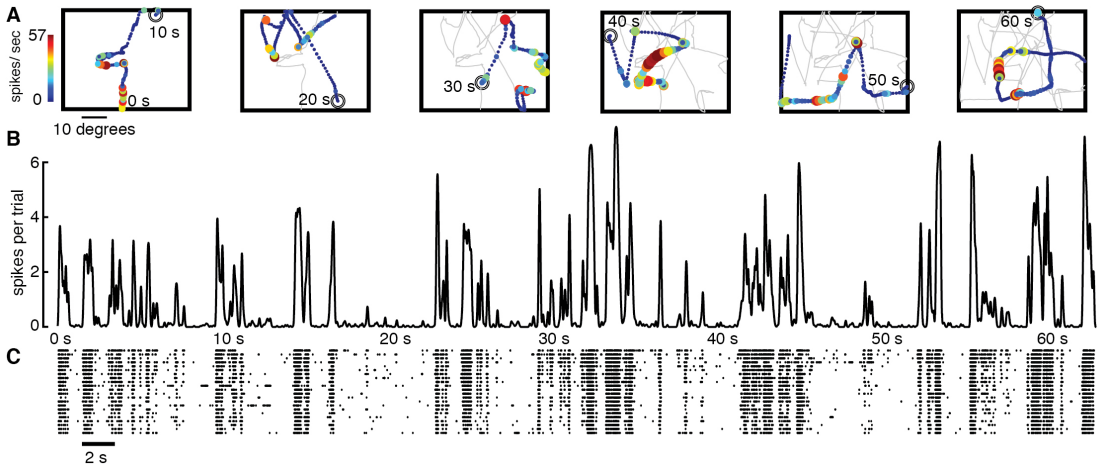


FIGURE 4.2.

Response of a single nerual unit in the brain of the jumping spider to prey-like movements of an artificial target. (A) A prey-sized black target (1.5 degrees) was shown moving across a white screen at various directions and velocities (see video S4.1). Each dot indicates the position of the target for each frame of the video (displayed at 25 frames per second). The color and size of each dot indicates the average firing rate over the 40 ms of each video frame (see B and C for histogram and rasters) with large, warm colored dots representing increased firing. Each box shows a 10 second interval with the sequence ending at the location highlighted by a black circle, target path taken in previous 10 second intervals are shown in grey. Note that target velocity is not constant. (B) Line-histogram (smoothed with a Gaussian filter [15]) showing the overall responses across 22 presentations of the 64-second stimulus. (C) Raster of spike times for each trial. Note the consistency in firing pattern from trial to trial over the entire experimental period of 24 minutes.

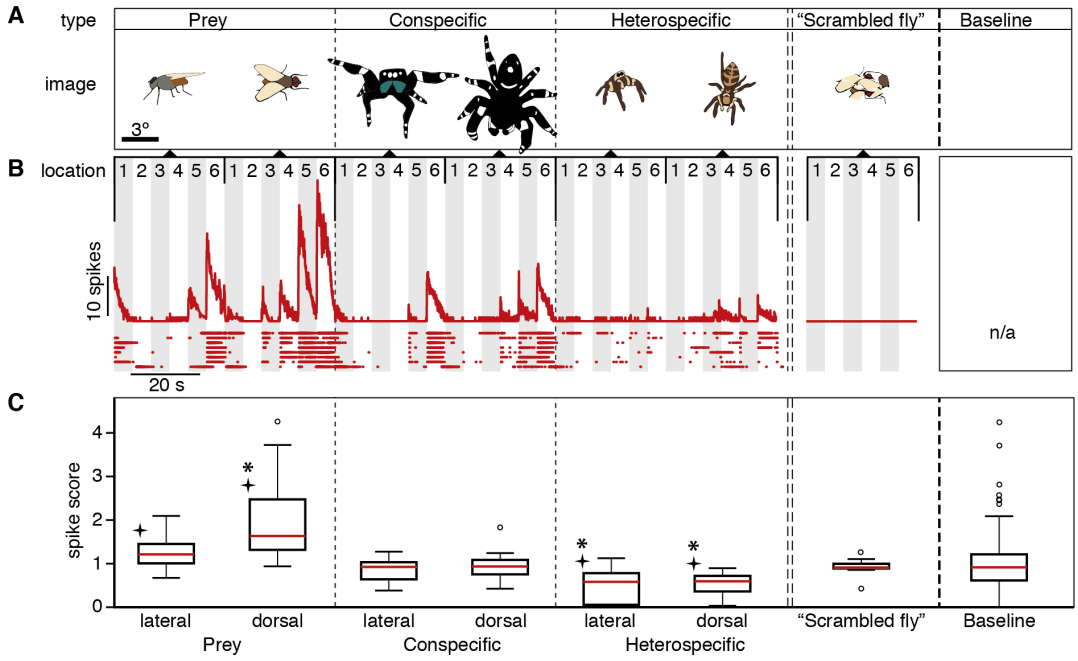


FIGURE 4.3.

Response to ecologically relevant images.

(A) Representative drawings of images used to test responses to potentially salient objects. (B) Responses of a single unit to images at 6 distinct horizontal locations for 8 trials. Rasters show spike times, while histograms were constructed as in Figure 4.2A. Due to the preference for the dorsal view of the fly, a scrambled fly image was constructed and tested, preserving all parts of the image but destroying its holistic identity. Presentations of the scrambled fly were interleaved with presentations of the standard stimuli and generated no response. (C) Summary of responses from unit shown in (B) across all trials (standard images n = 20; scrambled n= 14) coded as a spike score (see Supplemental Methods §X). Red line shows median score, boxes extend to the 25th and 75th percentiles of the data, while whiskers extend to cover 99th

percentile of a normal distribution. Data points outside this range are shown as circles. Overall recording time was over 5 hours, during which there were slight fluctuations in the overall firing rate. Y-axis indicates score of firing over baseline (see Supplemental Methods §X for calculation of overall baseline firing), hence the decision to code neural responses as a spike score. Crosses denote responses that were significantly different from the baseline, while asterisks denote responses that were significantly different from the response to the scrambled fly (Kolmogorov-Smirnov test, $p<0.05$ after Bonferroni correction for multiple-tests). Firing rates for the intact dorsal view of the fly were significantly higher than both baseline and scrambled rates.

2. Ecologically Relevant Images

The anatomical structure of jumping spiders' principal eyes should allow them to detect minute variations in target appearance [18], and behavioral studies have shown that they respond differentially towards objects displayed on a video screen [19]. We therefore presented dorsal and lateral images of a fly (potential prey), a conspecific jumping spider (potential mate or rival), and a heterospecific jumping spider (potential prey or rival) (Figure 4.3; video S4.3). Each image was sized to preserve the natural angular dimensions of the object (Figure 4.3A) and was shown at six locations on the spider's visual horizon (Figure S4.3A). The response of a single unit is shown in Figure 4.3. This unit showed a preference for dorsal images of the fly located on the right side of the screen (locations 5 and 6; Figure S4.3B [for a simultaneously recorded second unit with similar response, see Figure S4.3C-D]). The dynamic nature of the neural response to these images is best appreciated by viewing a video of the experiment that generated the data in Figure 4.3A-C; see videoS4.3. As a control, we presented a scrambled version of this preferred image that retained the size and contrasting features of the fly while destroying its figural integrity; a method typical of experiments on face recognition in wasps [20] and primates [21] [22]. Presentations of the scrambled image were interleaved with the original stimuli. Over a total recording time of 5 hours, responses to individual stimuli were normalized by the baseline firing rate for each trial to facilitate statistical comparisons (Figure 4.3C) (see Supplemental Methods §X for details). The response to the intact fly image was significantly greater than the response to the scrambled image

(Figure 4.3C; KS test, $p<0.05$ after Bonferroni correction for multiple tests; see video S4.3). A different unit in this spider, as well as a unit recorded from another spider, exhibited similar response patterns (Figure S4.3).

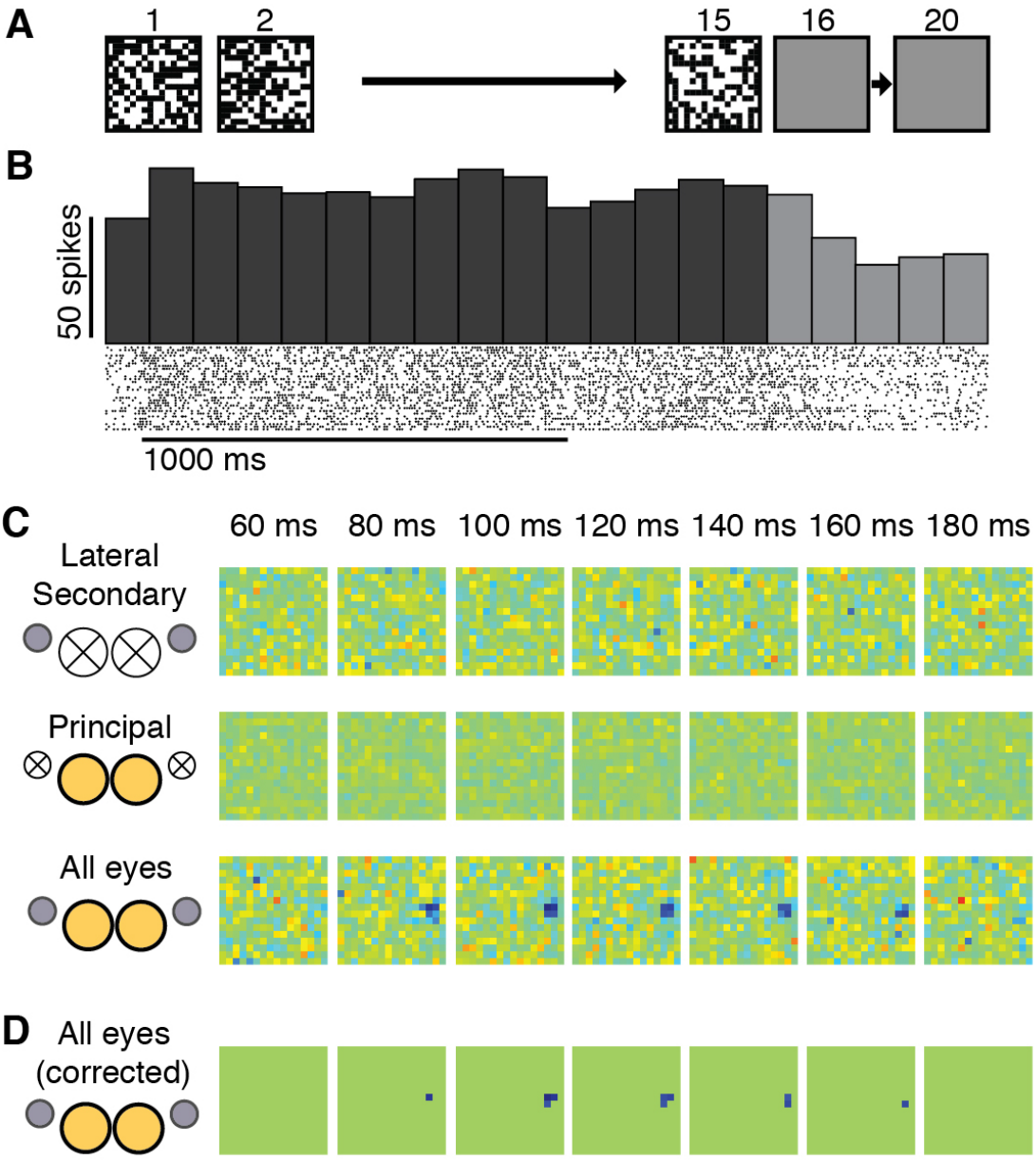


FIGURE 4.4.

Spatiotemporal receptive fields (STRFs).

(A) Stimuli were sequences of 16x16 grids of black and white checks. Each sequence was made up of 15 frames, with each frame presented for 100 ms

and each 15-frame sequence separated from the next by 500 ms of solid gray (50%) as an internal control (see Supplemental Methods §XI for details).
(B) Typical summary statistics for a single unit after spike sorting, with spike rasters (below) and a histogram of spike times in 10 ms bins (above). Note typical drop in firing rate when gray frames are present (light bars) relative to the response to the checkered frames (dark bars).
(C) Spike times and check contrast at each location were reverse correlated to generate STRFs for each eye treatment. Blue hues denote correlation with black checks, red denotes correlations with white, and green no correlation with either. Top: secondary eyes only (principal occluded); middle: principal eyes only (secondary occluded); bottom: all eyes intact (none occluded).
(D) STRF for the all-eyes condition, thresholded for significance at $p<0.05$, falsediscovery- rate corrected (see Supplemental Methods §XII). This was the only condition with a statistically significant response—significant STRFs were not observed for the conditions where the principal eyes or secondary eyes were occluded.

3. The Interaction between Principal and Secondary Eyes is Nonlinear

One of the most unusual features of salticid vision is the separation of visual tasks between anatomically distinct eyes. Of the two sets of forward-facing eyes, high acuity vision is the domain of the large principal eyes [18] while motion-detection is largely undertaken by the smaller secondary eyes (Figure 4.1A) [23] [24] [25]. We dissociated primary from secondary visual input by selectively placing eye occluders in front of each set of eyes, a simple but reversible procedure (see Supplemental Methods §V). We then deployed our third stimulus protocol, a white-noise-like stimulus that allowed us to identify spatiotemporal receptive fields (STRFs), revealing preferences for specific locations within a visual field, as well as the time-dependent aspects of the neural response [26] [27]. The stimulus consisted of a series of fifteen frames of 16Å~16-pixel pseudo-randomly distributed black-and-white checks, with each frame appearing for 100ms (Figure 4.4A; see video S4.4 for example response and Supplementary Methods §XI for complete stimulus details). Sequences were separated by 500 ms of featureless gray (50%) screen, which served as an internal control (Figure 4.4B). After each 26-minute recording session, units were identified by spike sorting and the firing patterns of individual units were reverse correlated with the pattern of checks at every location on the screen. From this analysis two strong inferences were drawn. First, for a subset of recordings, distinct STRFs were recorded only in the unoccluded condition. This indicates a significant interaction between principal and secondary eyes. When either set of eyes was occluded, no STRF emerged (Figure 4.4C top, middle). However, when both sets of eyes were unblocked,

an unambiguous STRF emerged (Figure 4.4C bottom). Because our analysis performs a linear reverse correlation between the stimulus and the response, the lack of a STRF in either occluded-eye condition implies that there was no linear relationship between stimuli and responses when the secondary or principal sets of eyes were forced to function independently. However, when the eyes were allowed to work together, a clear linear linkage between the stimulus and the response was exposed (Figure 4.4C, D). Second, STRFs emerged only after a long delay between stimulus onset and neural response (spatial-temporal window from 80 to 160ms; Figure 4.4C). The response latency was statistically significant even after correcting for multiple tests using statistical False Discovery Rate (see Supplemental Methods §XII). The long delay between stimulus onset and response suggests that there are at least several synapses between the retinae and the central body, supporting our supposition that the recordings came from a higher-order unit in the visual system [12]. A total of nine recordings across 6 animals generated clear STRFs; of these only the cell presented in Figure 4.4 exhibited the discussed nonlinear interaction, while three others showed different patterns of interaction (see Figure S4.4, video S4.4, and Supplementary Methods §XII).

CONCLUSIONS

Our recordings represent possible neural correlates for well-known behaviors exhibited by jumping spiders. Salticids on the hunt detect and respond to moving, small-field visual targets and this behavior is reflected in our recordings from interneurons to small moving targets (Figure 4.2) [27] [8] [28]. Even more remarkable is the response of single units to a dynamically changing visual scene, in space and time. We uncovered dramatic nonlinear interactions between principal and secondary eyes: there were neurons that responded sensitively to a localized region of space under normal viewing conditions, but that had no detectable receptive field when either pairs of eyes were occluded (Figure 4.4). This spatial and temporal integration may enable the demonstrated behavioral ability of a salticid to detect and localize its prey before navigating a "best-route" through a 3-dimensional maze to position itself so it can pounce down upon its prey [3], as well as at the last stages of predation, when the prey is scanned by the spider's principal eyes just before it pounces [29]. The behavioral repertoire of salticid spiders includes other acts that seem more vertebrate-like than spider-like [1] [30]. Our findings open the behavioral world of jumping spiders to investigation with the powerful techniques of neurobiology. It should now be possible to perform a neuroethological analysis of processing in the brain of jumping spiders to unravel the mechanisms that underlie the remarkable visual behavior of one of Nature's truly charismatic little animals.

REFERENCES

1. Harland, D., Li, D., and Jackson, R. R. (2012). How jumping spiders see the world. In How Animals See the World: Comparative Behavior, Biology, and Evolution of Vision (Oxford University Press).

2. Nelson, X. J., and Jackson, R. R. (2011). Flexibility in the foraging strategies ofspiders. In Spider Behaviour (Cambridge University Press). Available at: http://dx.doi.org/10.1017/CBO9780511974496.003.

3. Tarsitano, M., and Jackson, R. (1994). Jumping Spiders Make Predatory Detours Requiring Movement Away from Prey. Behaviour 131, 65–73.

4. Elias, D. O., Land, B. R., Mason, A. C., and Hoy, R. R. (2006). Measuring and quantifying dynamic visual signals in jumping spiders. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 192, 785–797.

5. Crane, J. (1949). Comparative biology of salticid spiders at Rancho Grande, Venezuela. III. Systematics and behavior in representative new species. Zool. N. Y. 34, 31–52.

6. Elias, D. O., Maddison, W. P., Peckmezian, C., Girard, M. B., and Mason, A. C. (2012). Orchestrating the score: complex multimodal courtship in the Habronattus coecatus group of Habronattus jumping spiders (Araneae: Salticidae). Biol. J. Linn. Soc. 105, 522–547.

7. Bodner, M. R., and Maddison, W. P. (2012). The biogeography and age of salticid spider radiations (Araneae: Salticidae). Mol. Phylogenet. Evol. 65, 213–240.

8. Jackson, R. R., and Pollard, S. D. (1996). Predatory Behavior of Jumping Spiders. Annu. Rev. Entomol. 41, 287–308.

9. Blest, A. D., Hardie, R. C., McIntyre, P., and Williams, D. S. (1981). The spectral sensitivities of identified receptors and the function of retinal tiering in the principal eyes of a jumping spider. J. Comp. Physiol. 145, 227–239.

10. Devoe, R. (1975). Ultraviolet and Green Receptors in Principal Eyes of Jumping Spiders. J. Gen. Physiol. 66, 193–207.

11. Babu, K., and Barth, F. (1984). Neuroanatomy of the Central Nervous-System of theWandering Spider, Cupiennius-Salei (arachnida, Araneida). Zoomorphology 104,
344–359.

12. Strausfeld, N., and Barth, F. (1993). 2 Visual Systems in One Brain – Neuropils Serving the Secondary Eyes of the Spider Cupiennius-Salei. J. Comp. Neurol. 328, 43–62.

13. Oberdorfer, M. (1977). Neural Organization of 1st Optic Ganglion of Principal Eyes of Jumping Spiders (salticidae). J. Comp. Neurol. 174, 95–117.

14. Quiroga, R. Q., Nadasdy, Z., and Ben-Shaul, Y. (2004). Unsupervised spike detection and sorting with wavelets and superparamagnetic clustering. Neural Comput. 16, 1661–1687.

15. Paulk, A. C., Phillips-Portillo, J., Dacks, A. M., Fellous, J.-M., and Gronenberg, W. (2008). The processing of color, motion, and stimulus timing are anatomically segregated in the bumblebee brain. J. Neurosci. 28, 6319–6332.

16. Lettvin, J., Maturana, H., Mcculloch, W., and Pitts, W. (1959). What the Frogs Eye Tells the Frogs Brain. Proc. Inst. Radio Eng. 47, 1940–1951.

17. Nordström, K., Bolzon, D. M., and O'Carroll, D. C. (2011). Spatial facilitation by a high-performance dragonfly target-detecting neuron. Biol. Lett. 7, 588–592.

18. Land, M. F. (1969). Structure of the Retinae of the Principal Eyes of Jumping Spiders (Salticidae: Dendryphantinae) in Relation to Visual Optics. J. Exp. Biol. 51, 443–470.

19. Bednarski, J. V., Taylor, P., and Jakob, E. M. (2012). Optical cues used in predation by jumping spiders, Phidippus audax (Araneae, Salticidae). Anim. Behav. 84, 1221–1227.

20. Sheehan, M. J., and Tibbetts, E. A. (2011). Specialized Face Learning Is Associated with Individual Recognition in Paper Wasps. Science 334, 1272 – 1275.

21. Tsao, D. Y., Freiwald, W. A., Knutsen, T. A., Mandeville, J. B., and Tootell, R. B. H. (2003). Faces and objects in macaque cerebral cortex. Nat. Neurosci. 6, 989–995.

22. Tanaka, J. W., and Farah, M. J. (1993). Parts and wholes in face recognition. Q. J.Exp. Psychol. Sect. A 46, 225–245.

23. Zurek, D. B., Taylor, A. J., Evans, C. S., and Nelson, X. J. (2010). The role of the anterior lateral eyes in the vision-based behaviour of jumping spiders. J. Exp. Biol. 213, 2372–2378.

24. Land, M. F. (1969). Movements of the Retinae of Jumping Spiders

(Salticidae: Dendryphantinae) in Response to Visual Stimuli. J. Exp. Biol. 51, 471–493.

25. Duelli, P. (1978). Movement Detection in Posterolateral Eyes of Jumping Spiders (evarcha-Arcuata, Salticidae). J. Comp. Physiol. 124, 15–26.

26. Hu, Q., and Victor, J. D. (2010). A set of high-order spatiotemporal stimuli that elicit motion and reverse-phi percepts. J. Vis. 10.

27. Land, M. F. (1971). Orientation by Jumping Spiders in the Absence of Visual Feedback. J. Exp. Biol. 54, 119–139.

28. Zurek, D. B., and Nelson, X. J. (2012). Hyperacute motion detection by the lateral eyes of jumping spiders. Vision Res. 66, 26–30.

29. Clark, D. A., Fitzgerald, J. E., Ales, J. M., Gohl, D. M., Silies, M. A., Norcia, A. M., and Clandinin, T. R. (2014). Flies and humans share a motion estimation strategy that exploits natural scene statistics. Nat. Neurosci. 17, 296–303.

30. Dolev, Y., and Nelson, X. J. (2014). Innate Pattern Recognition and Categorization ina Jumping Spider. PLoS ONE 9, e97819.

31. Hill, D. E. (1975). The structure of the central nervous system of jumping spiders in the genus Phiddipus (Aranaea: Salticidae) M.S. Thesis. (Oregon State Univ., Corvallis,
Oregon.).

SUPPLEMENTAL METHODS

Contents:

I. General experimental methods

Adult and penultimate jumping spiders (Salticidae: Phidippus audax) were collected locally around Ithaca, New York, USA and individually housed in the laboratory in 9x9x13cm plastic arenas (Containables, US Acrylic, Inc., Northbrook, IL, USA) with a 12:12 light:dark cycle. Spiders were given a constant source of moisture and sustained on a diet of domestic crickets (Acheta domestica). All spiders used in thecurrent experiment were adult females. Prior to recording, spiders were cold anesthetized for the minimal

time necessary to limit mobility (typically 3 minutes at approximately -5ºC) and waxed in place using Kerr dental sticky wax (58ºC melting point, Syborn Kerr, Emeryville, CA, USA) on a specifically designed 3D-printed plastic platform (produced using an Asiga Pico, Asiga, Anaheim Hills, CA, USA) using a cool soldering iron (Antex model C, Antex (Electronics) Limited, Travistock, Devon, UK) with the voltage limited to 55V using a variable transformer (Powerstat type 3PN116B, The Superior Electric Co., Bristol, CT, USA). This platform was designed as a minimally invasive way to prevent movement during recordings while also keeping the electrode entry point accessible and preserving the animal's natural forward field of view. Because the current investigation focused exclusively on the anterior eyes, both sets of posterior eyes were occluded with dental wax.

II. Recording methods

Recordings were made by inserting a tungsten microelectrode (4MΩ; MicroProbe Inc., Gaithersburg, MD, USA) directly through a small hole made in the animal's cuticle. The location of the entry point was based on external morphological features on the prosoma (akin to the cephalothorax) of the spider that could be readily identified under a Leica Wild M3Z stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) with a maximum magnification of 800x. The hole was made using the tip of a 0.5 x 16mm sterile syringe (Medi-plus, Shanghai KDL MED. Co., Shanghai, China). Because spiders maintain high internal fluid pressures, sudden and catastrophic fluid loss has been reported by other researchers who have

attempted to dissect away the cuticle in preparation for recording. However, we were able to avoid this lethal result by making a minimally small hole rather than what is usually attempted—on the order of 100μm as opposed to creating a window to expose the nervous system. Our procedure caused only minimal fluid loss, presumably because the natrual clotting action of the internal body fluid was sufficient to prevent any drastic fluid loss. The microelectrode was then passed through this tiny hole, and directly into the body cavity. Pre-insertion orientation of the microelectrode was executed using stereotactic micromanipulators (MM-3, Narishige International USA, Inc., East Meadow, NY, USA). Once in place, a hydraulic microdrive with a digital readout (Model 607W, David Kopf Instruments, Tujunga, CA, USA) was used to advance the electrode with up to 1μm resolution. A second sharp tungsten electrode with the insulation removed was inserted into the opisthosoma (akin to the abdomen in other arthropods) to serve as a ground. Resultant electrical activity from the recording electrode was collected using an extracellular headstage (Model 1800 A-M Systems, Sequim, WA, USA) and amplified 10,000 times and filtered (100Hz-5000Hz bandpass, 60Hz notch) using a differential AC microelectrode amplifier (Model 1800 A-M Systems, Sequim, WA, USA). Activity was monitored using an oscilloscope (TDS 1002, Tektronix, Beaverton, OR, USA). The signal was digitized with an analog-to-digital converter (NI PCI-MIO-16E-1, National Instruments, National Instruments, Austin, TX, USA) fitted with a breakout box (NI BNC-2090, National Instruments, Austin, TX, USA). All recordings were made using the Spike Hound data acquisition software (formerly called g-Prime) on a

computer running Windows 7 (64-bit; Microsoft Corporation, Redmond, WA, USA). All recordings were done on an air table (Micro-G, Technical Manufacturing Corporation, Woburn, MA, USA) with a custom-built wire-mesh Faraday cage.

III. Histological methods

Locations of recording sites in the central nervous system were verified using green fluorescent dye (dextran, fluorescein, 3000MW, anionic, lysine fixable, Molecular Probes, Eugene, OR, USA). Before the experiment began, the recording electrode was coated in fluorescent dye as describen in [S1]. This dye-enhanced electrode therefore left a track along its path through the nervous tissue, allowing for subsequent visualization of the recording site. At the conclusion of a recording session animals were sacrificed by placing them in a freezer at approximately -5º. The central nervous system was later dissected and preserved in 100% EtOH after stepping it from 70%, 90%, through 95% EtOH for a minimum of 15 minutes at each concentration and then cleared using methyl-salicylate.

Once cleared and fixed, brains were imaged at the Cornell University Institute of Biotechnology Imaging Facility using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Jena, Germany). The recording sites were interpreted in through the anatomical work of Hill (1975) [S2] and Oberdorfer (1977) [S3].

IV. Presentation of visual stimuli

All visual stimuli were generated using MATLAB (The Mathworks, Natick,

MA, USA), and played back as standard digital video files. Salticids are well known to respond to stimuli presented on video screens [S4] [S5], thus visual stimuli were presented using a conventional LCD computer monitor (ViewPanel VE150m, ViewSonic, Walnut, CA, USA; 60Hz refresh rate) placed directly in front of the animal (see S4.1 for dimensions and setup schematic). Background brightness values were 35 cd/m^2 for the prey-like moving targets and 18 cd/m^2 for the ecologically relevant images, while full black was 6 cd/m^2 and full white was 100 cd/m^2 (as in the receptive field and canonical cell characterization stimuli). A Visual Basic (Microsoft Corporation, Redmond, WA, USA) video player was designed and written to enable synchronization of stimuli start times with neural responses by generating a voltage spike that was recorded in parallel with the neural recordings.

V. Eye occlusion method

For the subset of experiments that focused on how the central nervous system integrates signals between the functionally specialized forward-facing eyes it was necessary to reversibly occlude specific sets of eyes. This was done using one of two types of occludors: a set cut out of thick black construction paper, and a plastic set made using a 3-D printer (Asiga Pico, Asiga, Anaheim Hills, CA, USA). These occluders could be used to either block out the view of the central principal eyes, or the two more lateral secondary eyes while leaving the view from the central eyes unobstructed. A range of sizes were made and matched to each individual spider to ensure that the desired eyes were completely occluded without obstructing the other pair. A 45º mirror was

placed just anterior to the animal and viewed from above, using the rig stereoscope, such that the spider could be viewed head-on. This allowed for the precise placement of the occlude and verification that the forward view of the target eyes was completely obstructed without affecting the view from the other pair.

VI. Overall recording statistics

Recordings were made from 66 sites across 34 animals. Total experimental time was 131.4 hours (this is the total time that an animal was in the rig with recording devices in place), from which 20 hours of selected neurophysiological recording data were captured and analyzed.

Targets moving in a prey-like manner (i.e. Figure 4.2):

6 cells tested across 4 animals; 4 cells showed a response that was correlated with the stimulus (66.7%). Ecologically relevant stimuli (i.e. Figure 4.3): 15 cells tested across 12 animals; 8 showed a response that was correlated with the stimulus (53.3%); 2 cells showed statistically significant responses to one stimulus type (13%). Spatiotemporal receptive field stimuli (i.e. Figure 4.4): 15 cells tested across 12 animals; 9 showed a receptive field (60%). Eight cells were tested using both the ecologically relevant and the STRF stimuli, 3 showed correlated responses to both, 3 responded only to the ecologically relevant stimuli, and 2 showed no correlated response to either stimulus.

VII. Cell characterization stimuli design

To characterize the response of salticid visual interneurons we deployed four different stimulus protocols. We presented a standard stimulus set consisting of spatial contrast gratings, bars, and blocks to document visual sensitivity (Figure S4.2). Characterizing visually responsive neurons is typically done by measuring neural responses to variation in several visual stimulus parameters [S6]. Here we used four basic ones: orientation, object width, velocity, and contrast. Objects always moved perpendicular to their longest axis. To determine which combination of these parameters was "most-preferred" by a given neuron, we recorded and analyzed responses to each parameter in a step-wise fashion, such that only one parameter was modified in a given trial (i.e. once the preferred orientation was determined, this value was used when varying object width). Parameters were set in the following order: orientation, object width, object velocity, and contrast. Because work in other visual systems has found some neurons to be sensitive to the general type of object displayed (e.g. STMD neurons in dragonflies), this process was repeated using contrast gratings, single bars, and single squares. Default values (values used before a preferred value for a given visual characteristic was found) were: orientation = 0°, spatial frequency = 1.56°, temporal frequency = 108°/sec, and contrast = 100%. Stimuli within each stimuli-type/ visual character set were presented in a randomized order, with each set of parameter values appearing 10 times. Figure S4.2A shows a representative raster and line-histogram from a unit responding to a bar shown moving in various directions. A rough receptive field could also be generated based on

these data by reverse correlating spike times with the onscreen position of the stimulus (Figure S4.2B), revealing that while this particular neuron was largely insensitive to the orientation, it did appear to be selective for a particular spatial location. Figure S4.2C shows the summary of responses to the full range of stimuli for the same unit presented in Figure S4.2A-B. This demonstrates that the visual neurons that we recorded from were responsive to canonical stimuli in manners similar to those of previously published visual systems [S7]. However, we emphasize that lacking any published information on salticid neuroanatomy, we had no expectations about the response properties of our recordings. Presumably, our recordings were made from higher-order visual neuropil, near the "central bodies," based on electrode placement relative to neuroanatomy of other spider species [S8]. Thus, our recordings were initially made without the benefit of extensive published work on salticid brain anatomy (see Hill [S2], Oberdorfer [S3]).

VIII. Cell characterization statistics

Each trial consisted of 10 repeats of each setting, presented in pseudo-random order. For each trial, values with the greatest mean spike rate were selected as "preferred." These "preferred" values were then used in the remaining trials.

IX. Ecologically relevant stimuli design

These stimuli were designed to probe for possible responses to higher-order visual tasks such as object discrimination. The image types were selected due to their ecological relevance: prey (a dipteran fly), a conspecific (possibly a

potential mate or rival P. audax), and a heterospecific (Salticus scenicus, a syntopic jumping spider species that is smaller than P. audax and could be a rival or possibly prey). Jumping spiders respond best to moving visual targets (and often ignore stationary ones) [S5] so each image appeared on the screen against a 50% gray background and moved, back and forth 10 times, over a 0.8° range for 5 seconds, then remained stationary for 1 second. Further, to ensure that the image was located in the field of view of a given neural unit, each image was displayed at 6 locations across the bottom of the screen (see Figure S4.3A), before the next image type was presented. In cases where sustained recordings were possible and a response for a single image (or image type) was found, further trials were run using modified versions of the preferred target. These generally consisted of "scrambled" versions of the image, (for example, the "scrambled fly" shown in Figure 4.2). The purpose of these controls was to better understand whether any increased neural activity was due to the "identity" of the object, or some lower level feature of the image, such as a preference for specific colors. This method of using a "scrambled" image follows common techniques used in other work on higher-order visual tasks such as face-recognition in mammals [S9] [S10] and wasps [S11]. Image manipulation was done using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). Images were modified based on versions obtained from the following sources:

fly side-view:

(http://www.redorbit.com/education/reference_library/science_1/insecta/2576174/housefly/)

fly top-view:

(http://www.ah.novartis.com/fhp/en/fly_species_house_fly.shtml)

conspecific head-on:

(http://bugguide.net/node/view/13237)

conspecific top-view:

(http://www.flickr.com/photos/saveena/1264357156/)

heterospecific head-on:

(http://www.flickr.com/photos/krypinaturen/6886351968/)

heterospecific top-view:

(Paul Shamble).

X. Ecologically relevant images statistics Firing rates in response to each stimulus image (Figure 4.3; Figure S4.3C,E), and stimulus location (Figure S4.3B,D,F) were analyzed in two ways: by comparison with a calculated baseline response and by comparison with the response to a "scrambled" image. The former sought to establish whether firing rates were correlated with specific images or locations, while the second sought to establish whether firing rates to "scrambled" images were significantly different from unscrambled ones under similar conditions. All analyses were corrected for multiple comparisons using Bonferroni correction and the comparisons were done using the Kolmogorov-Smirnov test.

Because recording times were usually long for these experiments (> 2 hours), baseline firing rates often changed across trials. A "neural response score" was therefore used to normalize each trial to enable inter-trial comparisons. Scores were calculated by comparing the firing rate in response to a particular stimuli

at a given location with the mean firing rate across that entire trial. Scores are multiplicative in nature—for example, a score of 2 is equal to a double the total median firing rate.

XI. Spatiotemporal receptive field (STRF) stimuli design

The stimulus from which the spatiotemporal receptive fields were constructed was specially designed to explore preferences for different kinds of motion [S12]. However, the stimulus was also designed to maximize randomness with respect to lower order visual properties–namely, contrast. The number of black and white checks that appeared at each location was roughly equal, making it a powerful method for analyzing the effects of contrast. Thus while the stimulus was designed to address multiple questions, the current study only addressed those results concerning the spatial-temporal effects of contrast sensitivity. The stimulus is reminiscent of the noisy "snow" that appears on a cathode-ray TV monitor disconnected from its signal, showing a set of pseudo-random black and white squares across the screen. Each static frame consisted of black and white checks arranged in a 16-by-16 grid. The height and width of each check subtended approximately 2.375^{o} of the animal's visual field. A checkerboard-like set of 15 frames was separated from the next set of 15 checkerboard frames by 5 frames of solid gray (50%). Each frame was presented for 100 ms (i.e. each set of checks were presented for 1.5 s with a 0.5 s break between sets). Each sequence was designed to depict a specific kind of motion. Twelve distinct types of motion and a purely random set were displayed, with each shown "moving" to the left and to the right.

Thirty-two repeats of each of these 25 unique sequences were presented in pseudo-randomized order. The total duration of the stimulus was approximately 26 minutes.

XII. Spatiotemporal receptive field statistics

In order to generate spatiotemporal receptive fields (STRFs), the contrast (black or white) of each check on the 16-by-16 grid was cross-correlated with the spike times. Repeating this cross-correlation process with different time delays and with specific intervals (the current study used 20ms increments and 20ms interval in each "panel") produced the temporal component of the result. See Figure S4.4 for complete results of the neural unit shown in Figure 4.4, and results from an additional unit. All results were corrected for multiple tests using the False Discovery Rate statistical method.

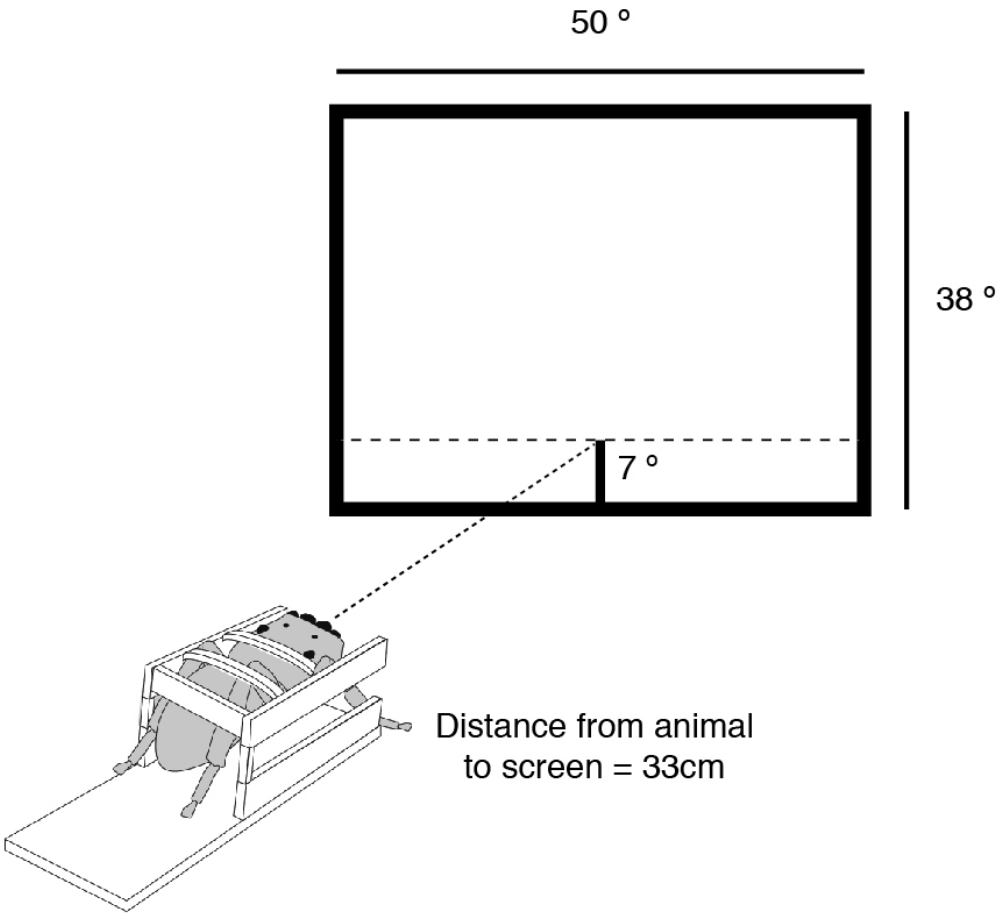


Figure S4.1. Schematic drawing showing basic experimental setup, including the angular dimensions of the LCD monitor used to present visual stimuli, related to Supplemental Methods. Spiders were restrained with wax in a 3D-printed holder placed 33cm from the LCD monitor. For a monitor of this size, viewed at the given distance, this provided a 50° by 38° area directly in front of the animal on which stimuli could be presented. The platform from which the spider viewed the monitor was slightly elevated such that 7° of the animal's view of the screen fell below the horizontal.

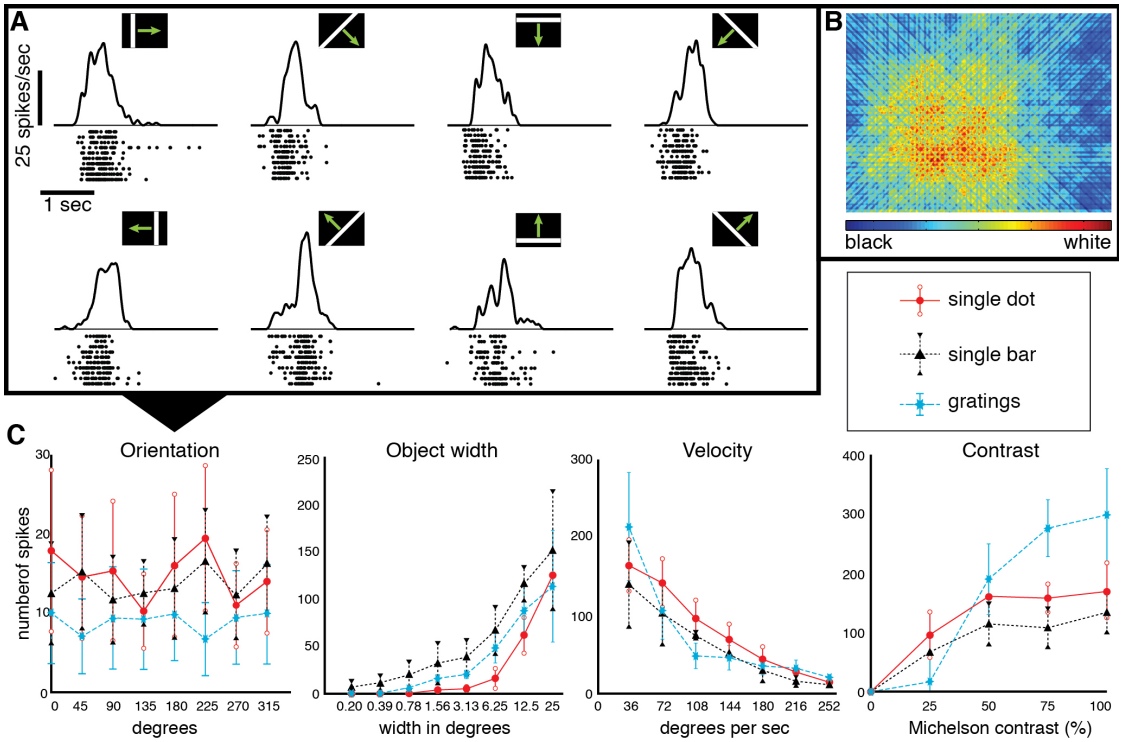


FIGURE S4.2. Response of neural units to canonical stimuli including gratings, bars, and dots, related to Supplemental Methods.

(A) Responses of a single unit to a white bar swept across a black screen in various directions (as depicted). Rasters show individual spike times for each trial (i.e. a single pass of the bar) while the line is a summary histogram of spikes across all trials, smoothed with a Gaussian filter [S7]. Stimuli were presented in pseudo-random order (see §VII).

(B) A receptive field generated by correlating normalized spike rate with the color on the screen. Warmer colors represent spikes correlated with lighter colors (i.e. the position of the bar).

(C) Summary of responses to gratings, single bars, and single squares for given orientations, object widths, velocities, and contrast values for the same neuron shown in (A,B). Stimuli for each parameter were presented step-wise

(i.e. orientation first, followed by width, velocity, and contrast) with established preferred values used when testing subsequent parameters. To allow comparisons between single object stimuli (bars and squares) and gratings, the spike number in response to grating stimuli were corrected by the number of cycles presented. For this neuron, responses to the three stimuli types (gratings, bars, squares) were similar, with no clearly preferred direction combined with a preference for large slow moving objects with high contrast.

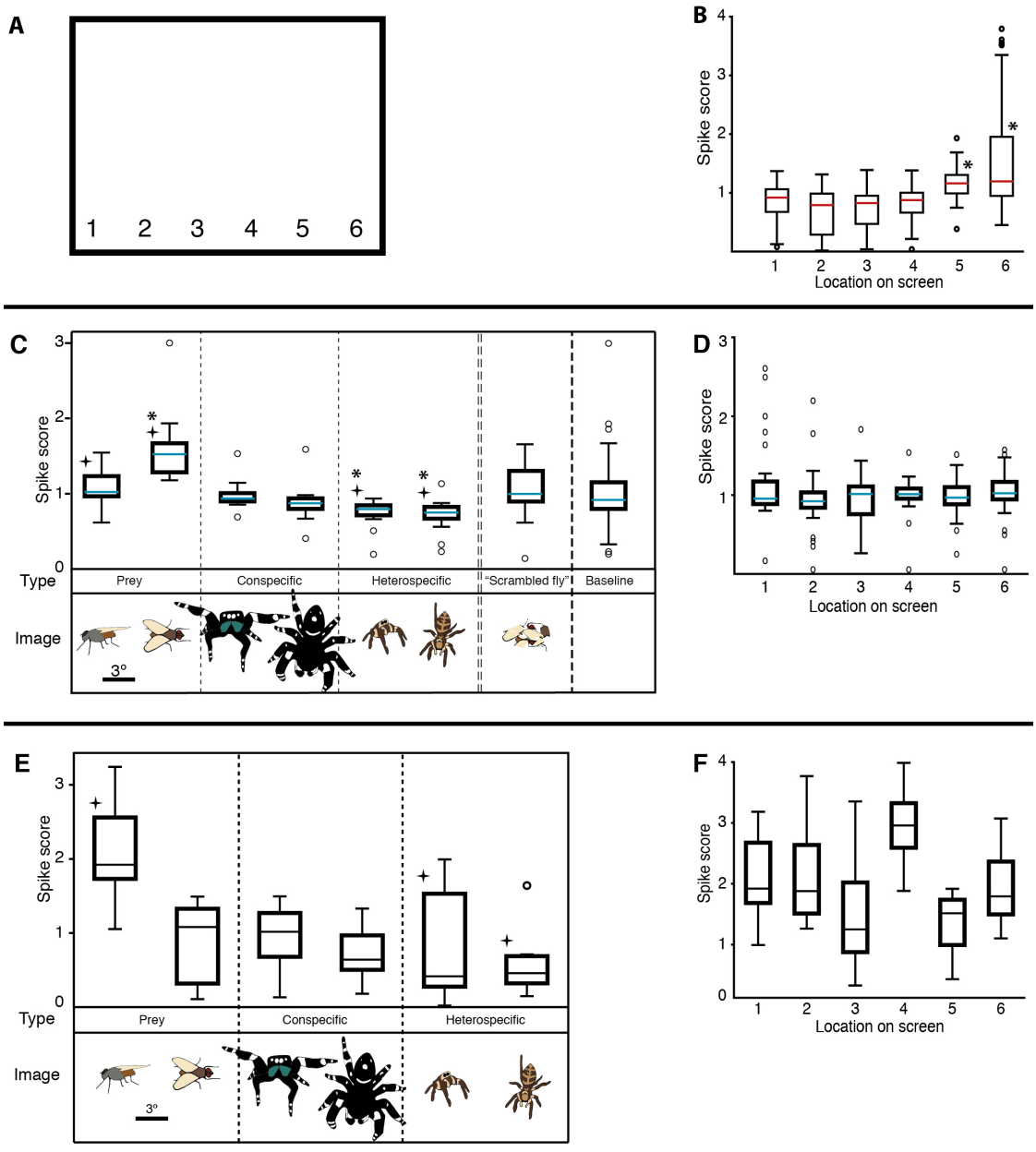
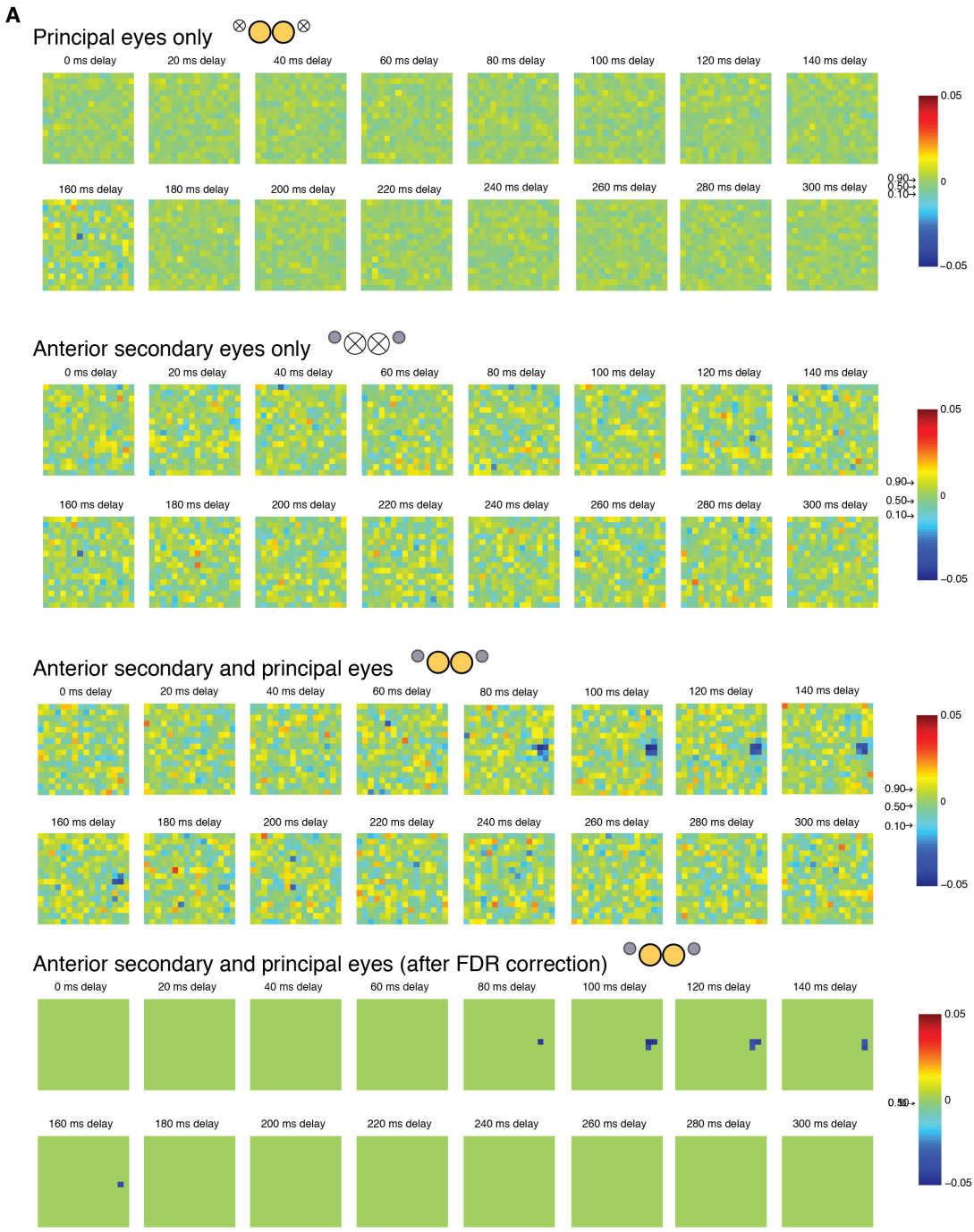


Figure S4.3. Neural responses to ecologically relevant visual stimuli, related to Figure 4.2.

(A) Schematic of the 6 locations where the stimuli appeared on the LCD

monitor. (B) Response of the neural unit shown in Figure 4.2 based on stimulus location. Lines at the center of each box represent medians; edges of each box are 25th and 75th percentiles; whiskers extend to 99th percentiles. Outliers beyond whiskers are shown as circles. Crosses denote locations that caused responses that were significantly different from the average firing rate (KS test; middle panel: 5th-position $p = 0.0006$, 6th-postion $p = 0.0022$).
(C) Results from a unit recorded simultaneously along with the unit shown in Figure 4.3 (the sorting of these two units is also shown in Figure 4.1B-D). Firing rate measured as a score of spikes per trial for three types of ecologically relevant images: prey,
conspecifics, and heterospecifics. All images preserved their relative sizes and are schematics of actual images presented. Crosses note responses that were significantly above or below the mean firing rate and asterisks show responses significantly different from the response to the "scrambled fly" (Kolmogorov-Smirnov test, $p<0.05$ after Bonferroni correction for multiple-tests; see Supplemental Methods §X). (D) Responses to visual stimuli at each of the 6 locations. No responses were significantly different from the baseline firing rate. (E) Results from a unit recorded in a different animal from that presented in Figures 4.3 and S3B-D, and showing a unit with a preference for an alternative prey image. (F) Responses to visual stimuli at each of the 6 locations. No responses were significantly different from the baseline firing rate.


A
Principal eyes only
0 ms delay
20 ms delay
40 ms delay
60 ms delay
80 ms delay
100 ms delay
120 ms delay
140 ms delay
160 ms delay
180 ms delay
200 ms delay
220 ms delay
240 ms delay
260 ms delay
280 ms delay
300 ms delay
0.05
0
−0.05
0.90
0.50
0.10
Anterior secondary eyes only
Anterior secondary and principal eyes
Anterior secondary and principal eyes (after FDR correction)

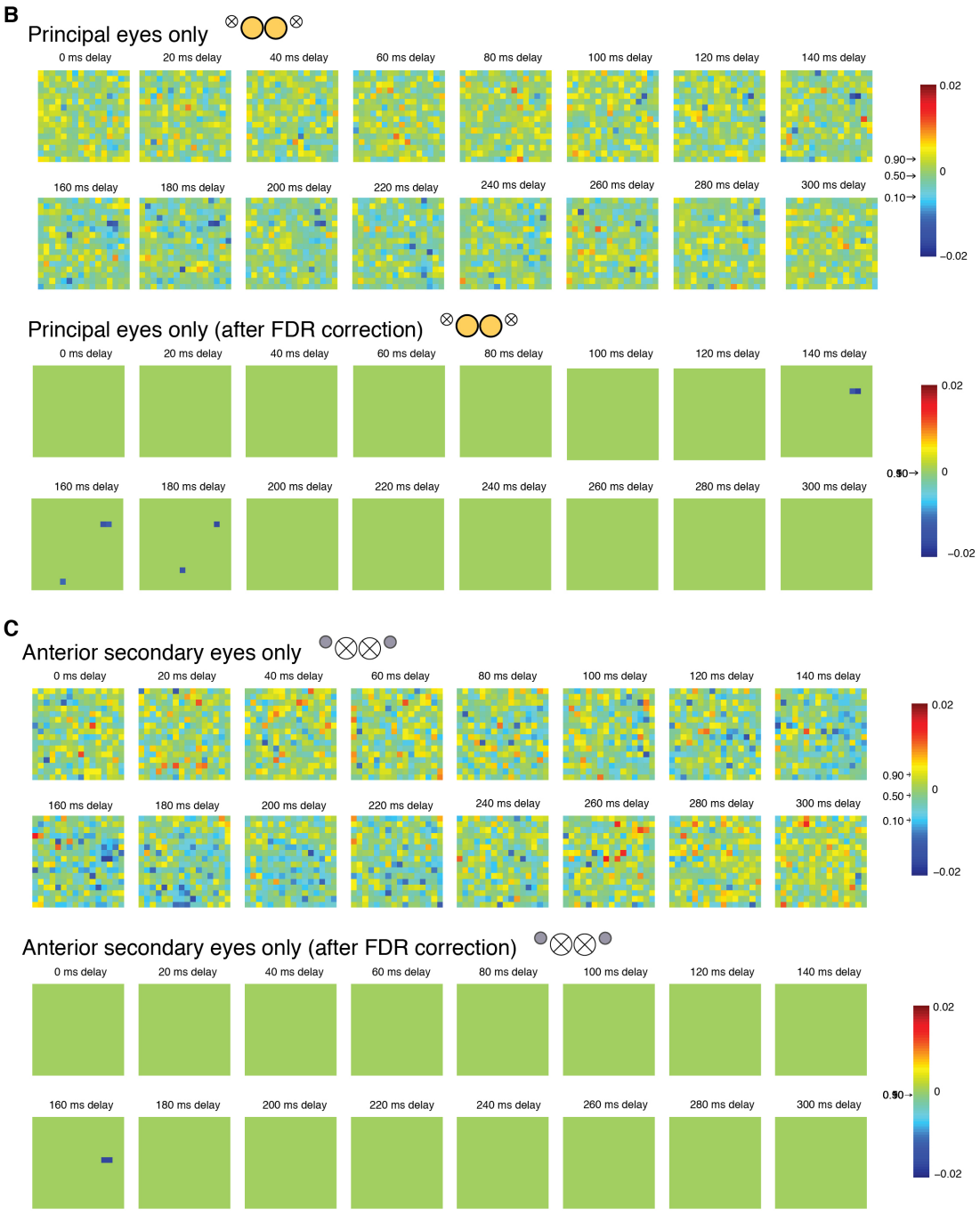

B
Principal eyes only
0 ms delay
20 ms delay
40 ms delay
60 ms delay
80 ms delay
100 ms delay
120 ms delay
140 ms delay
160 ms delay
180 ms delay
200 ms delay
220 ms delay
240 ms delay
260 ms delay
280 ms delay
300 ms delay
0.02
0.90→
0.50→
0.10→
0
−0.02
Principal eyes only (after FDR correction)
C
Anterior secondary eyes only
Anterior secondary eyes only (after FDR correction)

Figure S4.4. Spatiotemporal receptive fields (STRFs) of visually responsive units in the brain of the jumping spider Phidippus audax, related to Figure 4.4 Stimuli consist of a 16-by-16 grid of black and white checks, the pattern of which changed every 100ms. After recording neural responses, spike times were correlated with the color (white or black) of each check in the 16-by-16-by-t matrix, where t = total number of frames (see text and Supplemental Methods §XI-XII for a full explanation of stimulus design and statistical analysis). Cooler colors (blues) correspond to strong correlations between unit activity and the presence of black checks, while warmer colors (reds) are strong correlations between unit activity and the presence of white checks. Scale bars to the right of each set of panels depict the spread of data (90%, 50%, and 10% intervals) across a given trial.

(A) Complete results of from the experiment presented in Fig 4.4 In this

recording a receptive field was only present when both sets of eyes were allowed to work together, and this effect was still visible after correction for multiple tests using False Discovery Rate (FDR; bottom set).
(B-D) Results from a recording independent from the data shown in Figure 4.4 and section A of this Figure. This unit also showed clear receptive fields, however, unlike the unit shown in Figure 4.4, in this case receptive fields were present in all three eye treatments (B, principal only; C, anterior secondary only; D, both principal and secondary together). In all cases these receptive fields were statistically significant and can be seen even after FDR correction for multiple tests.

Figure S4.1. Schematic drawing showing basic experimental setup, including the angular dimensions of the LCD monitor used to present visual stimuli, related to Supplemental Methods. Spiders were restrained with wax in a 3D-printed holder placed 33cm from the LCD monitor. For a monitor of this size, viewed at the given distance, this provided a 50° by 38° area directly in front of the animal on which stimuli could be presented. The platform from which the spider viewed the monitor was slightly elevated such that 7° of the animal's view of the screen fell below the horizontal. FIGURE S4.2. Response of neural units to canonical stimuli including gratings, bars, and dots, related to Supplemental Methods. (A) Responses of a single unit to a white bar swept across a black screen in various directions (as depicted). Rasters show individual spike times for each trial (i.e. a single pass of the bar) while the line is a summary histogram of spikes across all trials, smoothed with a Gaussian filter [S7]. Stimuli were presented in pseudo-random order (see §VII).
(B) A receptive field generated by correlating normalized spike rate with the color on the screen. Warmer colors represent spikes correlated with lighter colors (i.e. the position of the bar).
(C) Summary of responses to gratings, single bars, and single squares for given orientations, object widths, velocities, and contrast values for the same neuron shown in (A,B). Stimuli for each parameter were presented step-wise (i.e. orientation first, followed by width, velocity, and contrast) with established preferred values used when testing subsequent parameters. To allow comparisons between single object stimuli (bars and squares) and gratings, the spike number in response to grating stimuli were corrected

by the number of cycles presented. For this neuron, responses to the three stimuli types (gratings, bars, squares) were similar, with no clearly preferred direction combined with a preference for large slow moving objects with high contrast. Figure S4.3. Neural responses to ecologically relevant visual stimuli, related to Figure 4.2. (A) Schematic of the 6 locations where the stimuli appeared on the LCD monitor. (B) Response of the neural unit shown in Figure 4.2 based on stimulus location. Lines at the center of each box represent medians; edges of each box are 25th and 75th percentiles; whiskers extend to 99th percentiles. Outliers beyond whiskers are shown as circles. Crosses denote locations that caused responses that were significantly different from the average firing rate (KS test; middle panel: 5th-position $p = 0.0006$, 6th-postion $p = 0.0022$). (C) Results from a unit recorded simultaneously along with the unit shown in Figure 4.3 (the sorting of these two units is also shown in Figure 4.1B-D). Firing rate measured as a score of spikes per trial for three types of ecologically relevant images: prey, conspecifics, and heterospecifics. All images preserved their relative sizes and are schematics of actual images presented. Crosses note responses that were significantly above or below the mean firing rate and asterisks show responses significantly different from the response to the "scrambled fly" (Kolmogorov-Smirnov test, $p<0.05$ after Bonferroni correction for multiple-tests; see Supplemental Methods §X). (D) Responses to visual stimuli at each of the 6 locations. No responses were significantly different from the baseline firing rate. (E) Results from a unit recorded in a different animal from that presented in Figures 4.3 and S4.3B-D, and showing a unit with a preference for an alternative prey image. (F)

Responses to visual stimuli at each of the 6 locations. No responses were significantly different from the baseline firing rate.

Figure S4.4. Spatiotemporal receptive fields (STRFs) of visually responsive units in the brain of the jumping spider Phidippus audax, related to Figure 4.4. Stimuli consist of a 16-by-16 grid of black and white checks, the pattern of which changed every 100ms. After recording neural responses, spike times were correlated with the color (white or black) of each check in the 16-by-16-by-t matrix, where t = total number of frames (see text and Supplemental Methods §XI-XII for a full explanation of stimulus design and statistical analysis). Cooler colors (blues) correspond to strong correlations between unit activity and the presence of black checks, while warmer colors (reds) are strong correlations between unit activity and the presence of white checks. Scale bars to the right of each set of panels depict the spread of data (90%, 50%, and 10% intervals) across a given trial. (A) Complete results of from the experiment presented in Fig 4.4 In this recording a receptive field was only present when both sets of eyes were allowed to work together, and this effect was still visible after correction for multiple tests using False Discovery Rate (FDR; bottom set). (B-D) Results from a recording independent from the data shown in Figure 4.4 and section A of this Figure. This unit also showed clear receptive fields, however, unlike the unit shown in Figure 4.4, in this case receptive fields were present in all three eye treatments (B, principal only; C, anterior secondary only; D, both principal and secondary together). In all cases these receptive fields were statistically significant and can be seen even after FDR correction for multiple tests.

Video S1. Neural responses to a target moving in a prey-like manner, related to Figure 4.2. The location of the target in each frame of the stimulus is shown as a dot where the size and color of the dot reflect firing rate (large, warm colored dots represent increased firing). Firing pattern data is summed across all 22 trials shown in Figure 4.2. Playback rate of this video matches the original playback speed of the stimulus. Video S2. An excerpt from a recording session showing response to the movingprey- like stimuli, related to Figure 4.2. Audio is an acoustic representation of the electrical signal recorded in response to the stimuli. Video S3. An exceprt from a recording session showing response to the ecologically relevant stimuli and associated "scramble" control, related to Figure 4.3. The audio is an acoustic representation of the electrical signal recorded in response to the stimuli. This is the same unit as in video S4 and complete results from this unit are summarized in Figure 4.3.

Video S4. An exceprt from a presentation of the checkerboard stimuli used to generate the spatiotemporal receptive fields as shown in Figure 4.4, related to Figure 4.4. The audio is an acoustic representation of the electrical signal recorded in response to the stimuli.

REFERENCES

S1. DiCarlo, J. J., Lane, J. W., Hsiao, S. S., and Johnson, K. O. (1996). Marking microelectrode penetrations with fluorescent dyes. J. Neurosci. Methods 64, 75–81.

S2. Hill, D. E. (1975). The structure of the central nervous system of jumping spiders in the genus Phiddipus (Aranaea: Salticidae) M.S. Thesis. (Oregon State Univ.,Corvallis, Oregon.).

S3. Oberdorfer, M. (1977). Neural Organization of 1st Optic Ganglion of Principal Eyes of Jumping Spiders (salticidae). J. Comp. Neurol. 174, 95–117.

S4. Clark, D., and Uetz, G. (1990). Video Image Recognition by the Jumping Spider, Maevia-Inclemens (aranea, Salticidae). Anim. Behav. 40, 884–890.

S5. Bednarski, J. V., Taylor, P., and Jakob, E. M. (2012). Optical cues used in predation by jumping spiders, Phidippus audax (Araneae, Salticidae). Anim. Behav. 84, 1221–1227.

S6. Hubel, D. H., and Wiesel, T. N. (1962). Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. J. Physiol. 160, 106–154.2.

S7. Paulk, A. C., Phillips-Portillo, J., Dacks, A. M., Fellous, J.-M., and Gronenberg, W. (2008). The processing of color, motion, and stimulus timing are anatomically segregated in the bumblebee brain. J. Neurosci. 28, 6319–6332.

S8. Babu, K., and Barth, F. (1984). Neuroanatomy of the Central Nervous-System of the Wandering Spider, Cupiennius-Salei (arachnida, Araneida). Zoomorphology 104, 344–359.

S9. Tsao, D. Y., Freiwald, W. A., Knutsen, T. A., Mandeville, J. B., and Tootell, R. B. H. (2003). Faces and objects in macaque cerebral cortex. Nat. Neurosci. 6, 989–995.

S10. Tanaka, J. W., and Farah, M. J. (1993). Parts and wholes in face recognition. Q. J. Exp. Psychol. Sect. A 46, 225–245.

S11. Sheehan, M. J., and Tibbetts, E. A. (2011). Specialized Face Learning Is Associated with Individual Recognition in Paper Wasps. Science 334, 1272 – 1275.

S12. Hu, Q., and Victor, J. D. (2010). A set of high-order spatiotemporal stimuli

that elicit motion and reverse-phi percepts. J. Vis. 10.

CHAPTER 5
SUMMARY AND FUTURE PLANS.

During my PhD I progressed from behavior, a study in basic classical conditioning to a very challenging neurophysiology. The classical conditioning part of my PhD was very fruitful and yielded-interesting results that was published and will impact the field of learning and memory in invertebrates. Later on, I learned how to use different technical and conceptual tools to address some of the more challenging questions in the acoustic and vision of jumping spiders and vision of dragonflies from the neurophysiology perspective.

Currently, at the time of writing this summary, our group is working on 3 future manuscripts from this recent work; the neuroacoustical behavior in jumping spiders, vision in jumping spiders and motion vision—a comparison of neural mechanisms for motion extraction between dragonflies and macaque.

In this work I have only reported the vision in jumping spider since it is in the most advanced stage of manuscript writing.

As for my future plans, I am staying in the Hoy lab to continue to work on neuroethology of spiders, dragonflies and wasps. Our group currently is composed of a few graduate students who are experts in their fields. We are taking advantage of this expertise and it is a model for my future career. I intend to be open to different future collaborations since we have perfected this kind of research approach such that we work very efficiently and fast to answer many unsolved questions in invertebrate neuroethology. The future

projects in the Hoy lab are very exciting, and we, as a team have many new open questions that we would like to try to answer.