An Unsupervised Method for Quantifying the Behavior of Paired Animals

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Abstract

Behaviors involving the interaction of multiple individuals are complex and frequently crucial for an animal's survival. These interactions, ranging across sensory modalities, length scales, and time scales, are often subtle and difficult to characterize. Contextual effects on the frequency of behaviors become even more difficult to quantify when physical interaction between animals interferes with conventional data analysis, e.g. due to visual occlusion. We introduce a method for quantifying behavior in fruit fly interaction that combines high-throughput video acquisition and tracking of individuals with recent unsupervised methods for capturing an animal's entire behavioral repertoire. We find behavioral differences between solitary flies and those paired with an individual of the opposite sex, identifying specific behaviors that are affected by social and spatial context. Our pipeline allows for a comprehensive description of the interaction between two individuals using unsupervised machine learning methods, and will be used to answer questions about the depth of complexity and variance in fruit fly courtship.

Keywords

animal behavior; courtship; unsupervised learning; computer vision; segmentation; *Drosophila melanogaster*; social behavior

1. Introduction

Social behaviors are exhibited by an extraordinarily wide range of animals. These behaviors, both mundane and elaborate, can be crucial for an animal's success in its natural environment. Observations of social behaviors have revealed patterns that are common across distant species, suggesting that certain patterns of action are evolutionarily favored and conserved [25]. For example, extravagant courtship sequences may have evolved because they signal a higher level of fitness while also playing a part in species recognition. [19,26].

There has been significant growth in the past decade in our ability to probe animal behavior with the goal of discovering and quantifying ever-smaller differences between phenotypes. Interest in genetic and neuronal control of behavior has accelerated the development of many recording and analysis techniques, and many groups have contributed to the efforts to track and identify individual and social behaviors in animals with the help of automation

[8,9,14,21–23]. We can now design experiments where single or multiple individuals are recorded over hours or even days, and each motion of the individual can be captured and compared [10, 20]. This kind of data can be used to estimate behavioral variety in an individual, and to make statistical comparisons across individuals, experimental contexts, strains, and species, as well as interactions between these individuals [12]. While these methods have greatly increased our understanding of the behavior of individuals, unsupervised techniques to enumerate and monitor the behaviors of socially-interacting individuals, including rare events that only happen in specific social contexts, are lacking.

One complex and oft-studied social interaction is male courtship in fruit flies. In textitDrosophila melanogaster, a male initiating courtship will position himself behind a female, orient towards her, tap her with his forelegs, and give chase. During courtship, the male deploys a variety of species-specific behaviors such as auditory cues like singing and abdomen drumming, as well as chemical cues such as those transmitted by licking and tapping. Eventually, if the female is receptive, copulation occurs [2, 6, 13, 24]. However, in spite of this somewhat predictable nature, courtship is highly variable between individual males, and the underlying basis of this variability is largely unknown [11]. One potential source of this variation in male courtship results from male-female interactions. Computational techniques have been used to track and quantify certain stereotyped patterns in male courtship behavior, such as chasing, wing displays, licking, and song [1,5]. However, in spite of decades of intense research into fly courtship, female courtship-specific behaviors, aside from that of slowing down in response to male song, remain largely undescribed [7]. Further, the ways in which female body movements, as opposed to their center-of-mass trajectories, contribute to variation in male courtship is completely unknown. To address these questions, we need more sophisticated methods to probe the behavior of socially-interacting animals.

In order to understand how male-female interactions shape courtship behavior we must be able to keep track of many potentially informative and computationally viable factors of the behavior of each of two interacting individuals across a large number of trials. Because these features can be difficult to score by eye, especially at the same time across multiple individuals, we must turn to automated methods to track and compare behaviors over time. Human observation is limited by factors such as definition bias and lapses in attention when observing many experiments over long periods of time. Supervised machine learning methods can be used to more accurately and fully observe complex behaviors for almost unlimited amounts of time but typically suffer from similar definition bias [15]. In order to create a link between subtle behavioral phenotypes and sexual selection, we need methods that can detect and define every possible behavior and quantify its dependence on specific environmental contexts. We currently do not know which behaviors or parts of courtship interactions are important for the emergence of complex traits, and require methods that can search the space of all behavior and eliminate human biases in order to find them.

Our group recently developed a method to catalog and quantify behavior in the form of a two-dimensional histogram of postural dynamics [4]. This method is used to organize and visualize the postural movements of an animal in an unsupervised manner. One complication that occurs when attempting to use this method with interacting animals is the mutual visual

occlusion that occurs when animals touch and their bodies overlap in the field of view. For courtship experiments in particular, the male and female flies exhibit many behaviors where they touch, including licking, tapping, and mounting. Here, we describe how we overcame this problem with computer-vision software that carefully handles the tracking and segmentation of individuals over the course of a multi-fly movie. Specifically, we introduce a method for tracking and segmentation that preserves identities and as much of the fly bodies as possible, even when imaging views are obstructed due to interaction. This method allows us to generate a behavioral description based on the behavior of both individuals. In the case of courtship, this provides a basis of comparison between male and female behavior. We demonstrate this method by finding subtle behavioral differences, in both males and females, between the case when a fly is isolated and when it is placed in a courtship context. We describe the emergence of behavioral programs based on the relative distance and orientation between two courting flies. Additionally, we examine the particulars of the context in which a behavior is performed, with the goal of constructing better models of courtship.

2. Behavioral Analysis Methods

Our group previously introduced an unsupervised method for discovering and cataloging the behaviors of individual animals using high-resolution movies [4]. For courtship, the problem is more complex: we wish to study the joint behaviors of two interacting individuals recorded in a single movie, tracking both what the animals do and how their actions influence each other. The first step in analyzing interactions is to track and segment the two fly bodies from each other to create a movie for each individual (§2.1–2.3). This allows us to monitor the behavior of each animal independently in addition to other parameters such as their relative positions and orientations (Fig. 1b). These time series, which capture both behavior and context, can then be used to study behavioral interactions (§3.1,3.3). The code and a sample embedding can be found at https://github.com/uklibaite/MultiFlyMapper.

2.1. Calculating Image Thresholds

Due to a backlit imaging setup, our video recordings produce a silhouette image of the flies where light is completely occluded by the body and only partially occluded by the wings and limbs. We performed all image operations on inverse images (light body on dark background) so that the highest intensity pixels reside in the fly bodies, medium intensity pixels make up the wings and limbs, and the background is low intensity. We calculated a low threshold T_F, which is higher than most of the background noise, to locate large connected components in each image. A higher threshold, T_B, was calculated to discern pixels that belong to the body (thorax, head, and abdomen) as opposed to the appendages. One concern in segmenting movies with multiple flies is that small lighting anisotropies and filming irregularities may disrupt segmentation during frames where flies touch. In order to successfully segment multiple flies from the same movie we calculated the body threshold value for each movie individually.

Before segmentation and tracking began, each entire movie was loaded into memory and 300 frames containing flies with completely separate silhouettes are sampled at random

from the movie. Each of the sample images is loaded individually and a three component Gaussian mixture model (GMM) is fit to the intensity distribution of all of the nonzero pixels that make up the image (Fig. 2). We used the Matlab function gmixdistribution.fit to estimate the means and mixing proportions of the three components using 10 replicates. At any given pixel value, we calculated the posterior probability of belonging to each of the three components. The posteriors were fit using a linear interpolation and the value at which a pixel is equally likely to be within the lowest- or middle-intensity component was found. This is the fly threshold T_F. The pixel value equally likely to be from the middle- or highest-intensity component is the body threshold T_B. We calculated T_F and T_B separately for each of 300 images sampled throughout the movie and the median threshold in each category is the final value used throughout the entire movie.

2.2. Assignment and Segmentation

To generate single-fly movies from paired-fly recordings, we need to segment the two flies from every image, assigning particular pixels to each fly, and track the motion of the flies over time. We do not address the issue of whether some pixels should be assigned to both flies in the case where their silhouettes overlap, and recognize that shared pixels could be assigned to both flies in order to improve tracking quality. We broke this problem up into three cases. For each case, we used different segmentation methods based on how difficult flies are to track and identify in any given frame (Fig. 3). In the first case the flies are clearly separated in the arena. We segment the flies through a simple thresholding and object tracking procedure. In the second case the flies are touching, but their bodies are still separable by applying a simple threshold. In this case, identification based on size and centroid is still possible, but some method for assigning pixels between the individuals is necessary. The third case includes frames where fly appendages and bodies are touching and therefore requires heuristic methods to assign the pixels between the flies. This is the most rare case by far and usually occurs during copulation attempts. Based on 15 movies of courting flies, corresponding to over 1 million frames, we find that Case I corresponds to ~ 90% of the frames, Case II to ~ 10% percent of the frames, and Case III to only ~ 0.25% of the frames.

We begin tracking at the first frame in which flies are easily separated. Fly identity is assigned based on body area. For courting flies, we assume that the female is larger and assign identity based on the area of the body in the first frame. We analyze each frame in series and a threshold is applied to produce a binary image (§2.1). If there are two connected-components from this threshold, T_F, with areas greater than 500 pixels (a value easily larger than any accidental matter in the dish but smaller than a single fly) then each of these connected components is used as a mask for an individual fly (Case I). If there is only one connected-component from this threshold, but two components when using the larger "body" threshold, T_B (Case II), we assign wing and limb pixels to each fly based on distance to the body components. We then produce a mask for each fly using the assigned pixels.

Finally, if there is only a single connected-component after thresholding with T_B (Case III), then we must segment the body and assign pixels in another manner. We first apply a binary distance transform to this connected component (using the bwdist command in MATLAB),

which contains pixels from both the male and female flies. Pixel values in this image correspond to the distance to the closest edge of the connected component. In most cases, this image contains two large basins, corresponding to each individual fly, separated by a noisy ridge. Because copulation attempts normally happen with the male behind the female, this prior knowledge is incorporated when identifying these basins as the fly bodies. A watershed transform is then used to find these two basins [18]. In practice, however, the basins are noisy and contain a number of small basins that get segmented separately in the watershed. We solve this problem by iteratively applying an H-minima transform (imhmin in MATLAB) with an increasing cutoff depth until there are a maximum of four regions segmented by the watershed. Two watershed regions are rarely found in this step because the male's posture often includes spread wings which produces extra regions. For this reason we group all but the largest watershed region into a single mask and in this way create two individual regions. Pixels surrounding the combined watershed basins above the threshold T_F are assigned to the different flies based on distance as in Case II. Since copulation is of great importance for the behavioral classification of courtship, this technique successfully tracks the flies even during highly occluded frames.

Assigning identity to each fly after the first frame depends not only on the size of each fly body but also on continuity from the previous frames. Each frame is considered both individually and in series so that prior centroid and identity information is always available. At times, segmentation distorts the body area of one or both flies, but assignment is maintained by assuming that the flies in a given frame will not travel far from their location in the previous frame and that fluctuations in body area are more likely than sudden changes to the fly position coordinates. The centroids and orientations of each fly in the original arena are recorded so that we can reconstruct behavior and analyze the effect of each measurement on the interaction behavior separately. Most tracking errors generated can be captured based on these assumptions.

2.3. Writing Aligned Movies

We create a mean, or basis, image of each fly body and wing silhouette for use in alignment and scaling of the individual movies. These basis images are generated for each of the flies before tracking and segmentation begins. The bases are binary images that indicate the median size and shape of the fly body after thresholding and alignment of the unique individuals. Basis images are useful because the paired flies are of different shapes and sizes and alignment is performed by matching a given fly image to an already aligned basis [4].

As we segment each frame and assign fly identities, we also align the images. At each time point, we record the orientation angle of the fly, which is the value of angular rotation necessary to produce the best alignment. This is also used as a way to check the consistency of alignment. The previous orientation angle is used to check and correct fly alignment during segmentation that distorts or clips part of the fly body, when the normal algorithm may fail.

Tracking and segmentation values are recorded in increments of 1,000 frames. If the flies have become impossible to segment for a large number of frames, meaning that the third case of segmentation has been used for a large number of frames, tracking is terminated as it

is assumed the flies have copulated. Each frame of the aligned movie for one of the interacting flies is 150×150 pixels and contains a centered and aligned fly. The area of the basis image for each fly body is calculated and used to scale each movie so that fly bodies for both flies consistently contain 1,500 pixels. This rescaling is necessary because all flies are slightly different in size and embedding onto the behavioral map will be more accurate when the final body shapes compared are as similar as possible. The two aligned and rescaled movies are saved separately as .avi files.

3. Results and Discussion

3.1. Behavioral Distributions for Male-Female Pairs and Isolated Flies

To identify behaviors that are specific to male-female interactions, we recorded separate movies of isolated males, isolated females, and male-female dyads in order to simulate potential courtship contexts. Each aligned movie, whether from an isolated or paired individual, contains fly images of a standard size and can be put through our behavioral analysis pipeline to generate behavioral density maps. The final format of the aligned fly data is the same for each fly regardless of whether it came from an isolated or paired experiment. This allows us to combine movies from both sexes and conditions in order to create a common 2-dimensional space of all behavior seen across all experimental conditions. We use the pipeline described in Berman et al to produce a single behavioral map that includes data from all isolated and paired flies. Briefly, this analysis includes dimensionality reduction of each image followed by a low-dimensional embedding of the temporal power spectrum [4] (Fig. 4). The behavioral map is a 2-dimensional histogram which provides a visualization of how much time flies spend performing any given behavior. The sharpness of certain peaks in the histogram indicates that some behaviors are more stereotyped than others.

As described previously, the behavioral map naturally divides into large scale regions of similar behavior which we operationally label by visual inspection [3, 4]. Here we split up the original 99 fine-grained behaviors found in our behavioral space by the watershed transform into seven course regions that we believe share similarities. These include locomotion, posterior behaviors such as rear grooming, anterior behaviors such antennal or eye grooming, actuation of the abdomen, and wing movements such as wing grooming and singing (Fig. 4b). These broad regions overlook much of the detail available in the map, but provide an entry point into making time-dependent comparisons of the behaviors of two interacting individuals. Ethograms, or time-dependent catalogues of the behaviors an individual performs, are produced by grouping points in the behavioral space as shown in Figure 4b and plotting the corresponding behavioral assignment as color over time (Fig. 4d). A supplemental movie shows the original and aligned behaviors, coordinates on the behavioral map, and ethogram outputs for each sex (Supplemental Movie). Parameters for setting the size of behavioral regions or clusters can be altered to address behavioral relationships at different scales over time, and to potentially locate behaviors that affect the outcome of courtship.

The embedding algorithm we use is stochastic, and produces a different map each time it is run, so we perform a single embedding using data from all individuals. A subsampling of

dimensionality-reduced data generated from videos of isolated males and females, as well as segmented males and females from the paired experiments was used to produce an initial behavioral embedding. Using many individuals guarantees that all behaviors present in our initial data, regardless of how rare, will be represented in our behavioral map. By subsampling the space of behaviors so that all actions we see from any context are represented in our original embedding, we can re-embed additional data onto this map without having to calculate a new embedding from the beginning. As we are interested in comparisons across many different contexts, embedding all data into the same map allows for direct comparisons of the probability density, or the frequency with which flies perform particular actions, between subpopulations of the data (Fig. 4c).

We recorded the behavior of 12 isolated females, 12 isolated males, and 15 male-female pairs. Comparing the behavioral distributions between contexts (isolated versus paired) reveals a number of features of male-female interaction (Fig. 4c). Overall, females spend more time exhibiting fast locomotion while males produce many more wing-related motions regardless of the presence of other sex. Difference maps between the isolated and paired condition for each sex show how behavior is affected simply by the presence of the courtship partner. We find that females run less and display more wing movements when a male is present. On the other hand, males not only produce more wing extensions when the female is present, presumably indicative of courtship singing, but also show subtle shifts to other wing- and abdomen-related motions. We use coarse behavioral descriptions here, but the same comparisons can be done at more specific regions on the map as well, and this method may be used to find the conditions in which subtle behaviors are enriched.

3.2. Behavioral Transition Probabilities for Male-Female Pairs and Isolated Flies

Using our system we can address some of the questions that have been asked about repeatable aspects of behavior, and if transition structure is one of them. [16, 17]. To visualize the differences in transition structure between experimental groups, we calculated transition probabilities between the fine-grained regions in our space as described in Berman et al [3] (Fig. 5). We consider transitions from one state to another within our fine-grained map, and ignore self-transitions. Occupancy in a behavioral state is defined to occur when a trajectory pauses at a regions in the behavioral-space for longer than a single frame. We plotted all transitions greater than 0.1 for all behavioral data from each specific context of interest (i.e. male in a courtship context) and find that there are qualitative differences in these plots. A comparison of the male transition structures between the two contexts shows that males paired with a female have a transition structure that is more highly inter-connected at nodes that denote wing and abdomen movements, suggesting that these motions are part of sequences initiated during courtship.

3.3. Behavioral Dependence on Distance and Orientation

We used the coordinates of the tracked flies to calculate the following parameters: the distance from the centroid of the male fly to the centroid of the female fly, d, the position angle of the female in a male-centered cartesian space, ϕ, and the position angle of the male in a female-centered space, θ (Fig. 1b). These three values are enough to describe the relative orientation and distance of both flies, and make it simple to find the spatial context

of a behavior from either individual at any point in time. We find that the behavior of both males and females varies as a function of the fly distance, d (Fig. 6a). When d is less than 3 mm, locomotion is underrepresented in both sexes. In contrast, wing, abdomen, and idle postures are much more likely. As distance increases ($3 < d < 6$ mm), overall locomotion at various speeds becomes more likely for the female while the male locomotes at a much higher relative speed. Due to the increase in locomotion, the likelihood of wing-, abdomen-, and idle motions diminishes. At larger separations ($6 < d < 9$ mm), the female spends almost all of her time locomoting whereas the male splits his time between fast locomotion and wing-based behaviors such as singing.

The behavior of each individual also depends on the relative orientation of their partner (Fig. 6b). We observe that the male extends his wings, and therefore presumably sings, more when he is behind the female than in any other angular quadrant. This may be due to the mechanics of song propagation and detection by the female or simply the drive to keep up with the female when she is moving away from him, as well as the fact that this geometric relationship is the most common across all movies of male-female pairs. The female, on the other hand, is more likely to be performing locomotory behaviors when the male is directly behind her. It is unknown whether these particular preferences are universal or specific only to this wild type strain of fly, and how these distributions vary with genetic background, context, and geometry. By exploring these differences and calculating enrichments in relationships across strains and species, future work will determine whether these may be important selected characteristics of courtship.

3.4. Fine Scale Dissection of Orientation Effects

We display fine behavioral resolution in another manner by creating probability density maps that describe the spatial organization of interacting flies during particular behaviors(Fig. 7). These plots reveal how the position of a partnered fly may selectively drive certain behaviors. The examples shown here are created by focusing on specific masked regions of the behavioral space, and then finding the spatial contexts in which those behaviors occurred. Each time an individual exhibits behavior in the masked region, the relative position of its partner is recorded.

We find that locomotion with wing movements and song-like wing extensions (rows 1 and 4 of Fig. 7) are much more common in males, and especially in paired males. In fact, these behaviors are minimal in unpaired males and the frequency with which they are performed by females is low regardless of the presence or absence of a male. The same tight localization pattern in which the female is positioned directly in front of the male during male song-like wing extension indicates that male wing motions, and wing extensions in particular, are potentially driven not only by sex and context of the behaving individual (male, and courtship), but also by the position of that individual's partner, the female. We also find that behaviors change in the paired female. Having a male present and courting induces an abdomen-bending behavior in the female (row 3 of figure Fig. 7). This action is also performed by the male, but its frequency is not dependent on the presence of a courtship partner. The relative spatial position of paired flies does not appear as crucial for abdomen related behaviors as it is for wing extensions. The paired females show a pronounced

increase in abdomen bending over unpaired individuals, suggesting that this behavior is important for the communication of receptivity or rejection by the female in a courtship context.

Finally, we see that even behaviors that are commonly displayed in both paired and unpaired conditions can still give insight about the mechanics of courtship-context interactions. While wing grooming (row 2 of Fig. 7) occurs in all contexts for both sexes, paired males and females both display an increase in this type of grooming over their unpaired counterparts. Further exploration of when grooming occurs, and in what spatial context, will aid in the understanding of how simple and common behaviors such as grooming may be employed to communicate during courtship. Comparisons with same-sex dyads can help further distinguish which male-female interactions are unique.

A compelling open question in behavioral science is one of how differences in complex traits arise from subtle changes in the genome. Equally compelling is the goal to understand how much of behavior is mediated by simple interactions with another individual, especially when these interactions are inevitable like in the case of courtship. We hope to use the method presented here to investigate these questions in fruit fly courtship by breaking down and quantifying this complex set of interactions. Here we have shown several ways to display behavioral differences between flies in different contexts. These methods are useful for interrogating courtship behavior in a principled manner when conducting research at the interface of genes, environment, and behavior.

4. Experimental Methods

4.1. Fly Stocks and Experimental Conditions

Wild type *D. melanogaster* flies were isolated on eclosion and aged four to six days before imaging. Females were housed in batches of up to 50 virgin females while males were individually housed in 96 well deep well plates sealed with microporous tape. All flies were raised and imaged at 21–22C with a 12H light on/light off cycle.

4.2. Fly Behavioral Assays

Our behavioral data is produced by filming silhouettes of a single or multiple flies from above in a circular chamber that is approximately 25mm across. Flies are prevented from crawling on the top of the chamber due to size restrictions and the application of a siliconizing reagent the day before filming in order to allow ample time for evaporation before performing experiments.

All behavioral experiments were filmed within 3 hours of the incubator light coming on. Single flies and courting fly pairs were filmed 12 at a time in three 4-camera setups after manual aspiration into individual arenas. Image data was acquired via a .5-inch Super-eXtended Graphics Array (SXGA) CMOS image sensor with an array of 1280 by 1024 high sensitivity pixels that measure 4.8 μm on a side. The sensor has on-chip 10-bit A/D converters and data is transferred via USB 3.0 to the data acquisition computer. Imaging was started approximately 5 minutes after introduction of flies, and flies were filmed

continuously for 30 minutes. BIAS capture software was used to produce 1024×1024 pixel frames of each walking fly or courting fly pair at 100Hz.

4.3. Preprocessing Movies and Coarse Fly Detection

In order to limit the size of files that are processed in series, and to begin the process of isolating touching flies, we first preprocess each movie by locating and saving the general region of the image where each fly is found in every frame. Each 1024×1024-pixel image from an input movie is subjected to coarse tracking to identify the objects. This process is completed in parallel by breaking up the long movie into short movies of 300 frames, where a threshold is applied to each frame and large connected-pixel components are discovered. If two separate connected objects are found, and their separation is larger than 100 pixels, a 150×150 region around each object is found and the regions are placed next to each other with blank space underneath to make up a 300×300-pixel image. If flies are separate but within 100 pixels of each other, a single 300×300-pixel image around the mean centroid of the flies is saved instead. If the image contains only one connected object then a single 300×300-pixel region around the centroid is saved. The short movies are recombined and the end result is a 300×300-pixel movie of the same length as the original movie comprised of either two single-fly images or a single double-fly image for each frame.

The location of each recorded region is saved to an informational text or .mat file as coordinates of the upper left and bottom right corner of each extracted image in the original movie frame. This file is used later in describing the center of mass motion of each individual. This text file also includes information about which configuration the frame was saved in as a reference during segmentation, based on whether one large or two smaller regions were saved. The preprocessed movie is saved as a .avi file and contains all of the information necessary, in conjunction with the informational .mat file, to reconstruct the original movie. This reduces the space requirements per movie, as well as creates a standard data type when using different acquisition software.

4.4. Copulation Detection

Tracking and segmentation continues until the bodies have proved impossible to separate for over 500 frames. This cutoff doubles as a coarse copulation detector, and signals the end of tracking for that particular movie. It is possible to continue tracking once copulation has ended, but we do not use these frames in our analysis because tracking may have to be resumed after a prolonged period when bodies are not viable for segmentation using our algorithm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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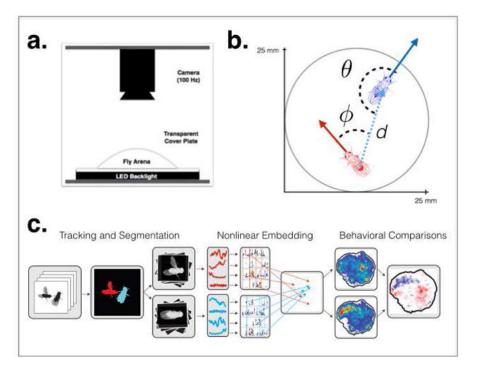26. Tinbergen, Niko. On aims and methods of ethology. Zeitschrift für Tierpsychologie. 1963; 20(4): 410–433.

Figure 1.
Experimental setup and analysis pipeline. a) Diagram of the recording setup used to take behavioral movies. b) Schematic of angles and distances that capture the relative orientation of the male (red) and female (blue). c) Pipeline of image analysis steps used to produce the joint behavioral distributions.
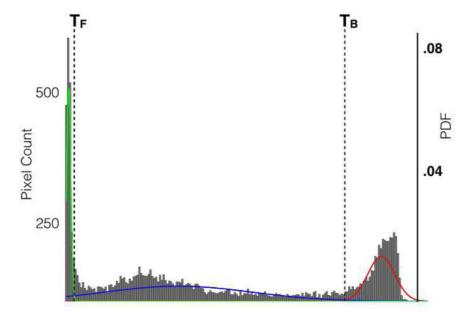
Figure 2.
Histogram of pixel intensities within fly connected components for one sample image. The probabilities of belonging to each component of the gaussian mixture model (GMM) are shows in red (low intensity), blue (medium intensity), and green (high intensity). The thresholds for the image are determined by finding the intersection of the posterior probabilities between two adjacent sets of pixels given by the GMM and are labeled with dotted black lines.
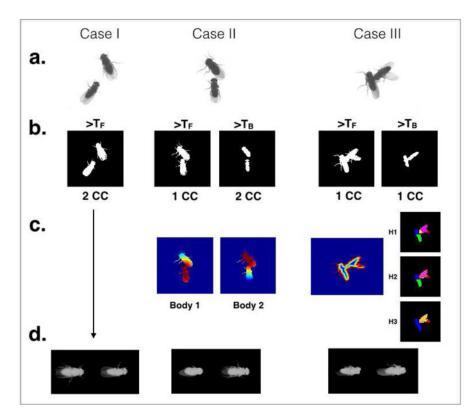
Figure 3.
Overview of algorithm used to create aligned movies of individual flies from original courtship movies. Examples of images created during particular steps in alignment and segmentation of a courting pair of flies depending on which heuristic was used. Case I: a) Original image, b) applying the lower threshold T_F produces two separate connected components, d) output images are aligned masks of connected components from panel (b). Case II: a) Original image, b) applying threshold T_F produces a single connected component, but isolated bodies of the two flies become apparent after applying threshold T_B, c) A gradient shows the distance of all post-threshold pixels to a given body mask. The pixels are assigned to whichever mask boundary they are closest to, d) output images are aligned masks of all pixels assigned to a given fly. Case III: a) Original image, b) thresholds T_F and T_B both produce images with a single connected component, c) the binary body image is produced by applying a watershed algorithm iteratively with increasing H-minima transform values until a maximum of four watershed regions are produced. The single largest region is assigned to a single fly mask while the other regions are grouped to produce the second fly mask, d) output images are aligned masks of all pixels assigned to that particular fly.
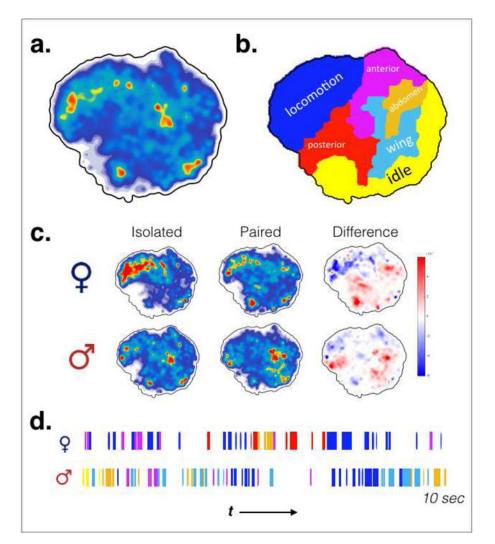
Figure 4.
Behavioral maps produced from Canton-S wild type flies in both isolated and courting contexts (male-female dyads). a) A density map containing all movies in the dataset (paired male, paired female, isolated male, isolated female). b) Coarse descriptions of map regions based on visual inspection of the aligned movies. c) Behavioral density maps created by plotting only points produced by courting males, isolated males, courting females, isolated females, and the differences between the isolated and paired contexts. Difference maps indicate which behaviors are enriched during courtship (red) or when individuals are isolated (blue). d) Simultaneous ethograms for a single ten-second bout of interaction with colors corresponding to the coarse labels in panel (b). Colored labels are assigned to each frame only if the behavior is stereotyped, as defined in Berman et al., resulting in white space during transitions and unstereotyped behaviors. [4].
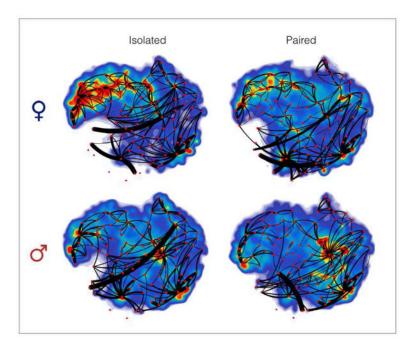
Figure 5.
Transition probabilities between fine-grained behaviors are plotted over behavioral density maps for males and females in both the paired courtship-context and the isolated context. Red points denote peaks in local density, and we use 99 regions which resulted from our watershed of the density map. Transitions are drawn as lines from one behavior to another, where right-handed curvature shows the direction of the transition. Line thickness represents the probability of transition given the fly is in the initial behavioral state, and only transition probabilities greater than 0.1 are plotted for clarity.
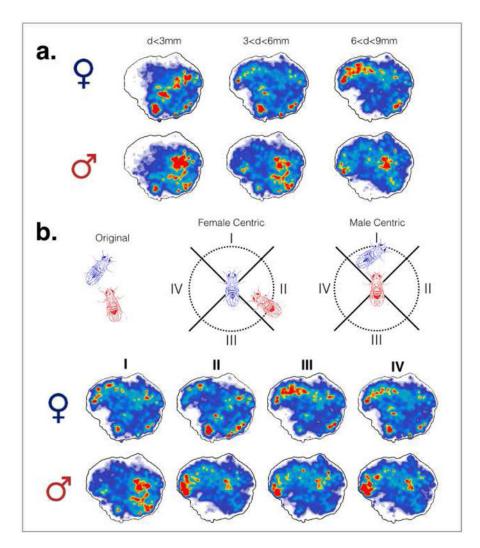
Figure 6.
Behavioral map densities for a variety of spatial contexts. a) Male and female behavioral density maps plotted for three intervals of the distance *d* between paired flies (< 3 mm (top), 3–6 mm (center), and 6–9 mm (bottom)). b) A schematic of distinct angular quadrants shows how flies may be viewed in the female- and male-centric spaces. Each row of behavioral maps corresponds to the behavioral density of the given sex while the other fly's angular position is in a given quadrant.
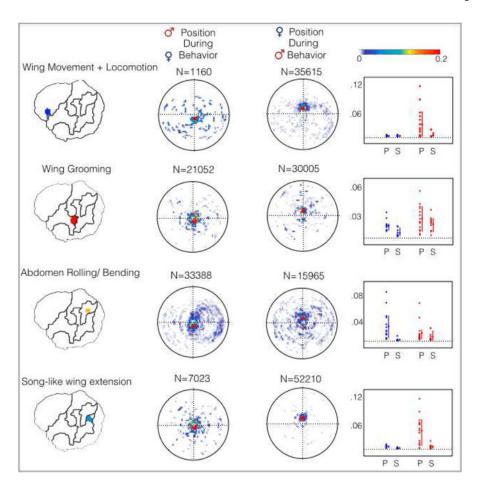
Figure 7.
A panel of sample behaviors displaying statistics on the location and orientation of each fly when the behavior is performed relative to its partner, as well as the total fractional occupancy in the male (red) and female (blue) during paired (P) and single trials (S). Each row describes the density of occupancy in space of the partnered fly while the specified individual performs a given behavior. The density in the center panels can be read using the color bar at the far upper right, while points in the far right panel correspond to density of the behavior per individual in the corresponding context. Error bars show the standard error of the mean for each set of points. Behaviors specified are a) Fast locomotion with some wing involvement, b) rear and wing grooming, c) abdominal rolling or bending, and d) song-like wing extension.