Plasticity in expression of fruit fly larval feeding clusters in response to changes in food quality and distribution

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Abstract

Joining a social group entails a range of possible costs and benefits, with the balance of pros and cons potentially dependent on the specific conditions present in the local environment. In the third instar stage of fruit fly (*Drosophila melanogaster*) development, individuals may join together into foraging groups that can increase access to buried food resources, but this collaboration comes with the possible risks of kleptoparasitism or slowed development. Cluster feeding in *D. melanogaster* larvae has the potential to be a valuable model for studying the dynamics of social and group behaviours, but little is currently known about the plasticity of its expression. In this study we set out to explore how this collective behaviour might be shaped by the nutritional quality of the environment and/or the spatial distribution of resources by manipulating the nutritional quality of the larval environment and the configuration of available resources. By tracking the timing, frequency, composition and size of any feeding clusters that subsequently developed we could better understand the factors that influenced the expression of this social behaviour. We found that cluster expression varied temporally and with the type of resources present in the environment. When possible, larvae formed clusters more frequently to take advantage of otherwise inaccessible resources. The increased clustering patterns were most dramatically manifested as deeper feeding mines. This work expands our understanding of the plastic nature of this collective behaviour, given that discrete environmental changes elicited dynamic changes in larval behaviours.

Keywords: food preferences, clustering, cooperative behaviour, Drosophila melanogaster, social behaviour, cooperative foraging

Introduction

Group living is a common phenomenon that is seen in a wide range of taxa and exhibits both great diversity and flexibility in expression (Krause & Ruxton, 2002). The requirements for animals to be considered part of a social group are that individuals are i) located in close proximity, ii) stay together for a meaningful period of time, and iii) come together for 'social' reasons (*i.e.* not because each was independently attracted to some external stimulus; Pitcher, 1983; Navarro & del Solar, 1975; Simon et al., 2012; Burg et al., 2013; Ramdya et al., 2017; Guo et al., 2017). There are many potential benefits of joining a social group, including increased access to resources, improved mating opportunities, and protection from predation (Giraldeau, 1984; Krebs & Davies, 1996; Krause & Ruxton, 2002; Majolo et al., 2008; Pike et al., 2008; Ramdya et al., 2017; Nagy et al., 2020). At the same time, group living may come at the cost of increased visibility to predators, heightened intra-specific aggression, and the faster depletion of resources as competition intensifies with group size (Krebs & Davies, 1996; Majolo et al., 2008; Ioannou et al., 2019). To understand the evolution and variability of the expression of this type of social behaviour, we must explore how individuals weigh the costs against the benefits of group living.

Fruit flies, *Drosophila melanogaster*, have been an important model species in countless genetic, behavioural, and evolutionary studies (reviewed in Bellen et al., 2010; Brookes, 2002) but have not historically been considered a "social" species (Chen & Sokolwski, 2022). However, that perception has begun to rapidly change and there is an increasing recognition that fruit flies engage in a number of collective behaviours (Ueda & Kidokoro, 2002; Durisko et al., 2014; Lone & Sharma, 2011; Durisko & Dukas, 2013;

Schneider & Levine, 2014; Siva-Jothy & Vale, 2019; Rooke et al., 2020, Chen & Sokolowski, 2022). Most studies to date have focused on the actions of groups of adults as they frequently aggregate in order to forage, to mate, and when laying eggs (Shorrocks, 1972; Sarin et al., 2009; Simon et al., 2012; Durisko et al., 2014; Lihoreau et al., 2016; Dombrovski et al., 2017; Ramdya et al., 2017). However, social behaviour is not limited to *D. melanogaster*'s adult stage and is also expressed in their larval stage(s).

D. melanogaster larvae are prone to aggregating (Durisko & Dukas, 2013), and use visual and pheromonal cues to locate each other (Mast et al., 2014; Wong et al., 2017). Doing so may increase their protection from predation and aid in communal digestion via secreted enzymes (Gregg et al., 1990; Prokopy & Roitberg, 2001; Sokolowski, 2010; Wu et al., 2003; Sakaguchi & Suzuki, 2013). However, larvae in close proximity may also risk cannibalism from their neighbors (especially if they are not close kin; Vijendravarma et al., 2013; Khodaei & Long, 2020; Fisher et al., 2021). Foraging is of prime importance to larvae, as in *D.melanogaster* — like other holometabolous insects (such as *Heliconuis cydno*, *H. charitonuis*, and *Dryas julia*, in Boggs 1981) — survival and adult body size are largely determined by the amount of resources that can be acquired during this phase of their life cycle (Sang, 1956; Boggs, 1981; Prasad et al., 2003). This objective underlies a complex social aggregation seen in the late third-instar phase, the formation of feeding clusters to access nutrients that are buried lower than can be reached by an individual (Figure S1). This "clustering" phenomenon (Dombrovski et al., 2017, Khodaei & Long, 2019) involves synchronized digging by groups of larvae to create an open-pit mine in the semi-liquified surface of substrate which has become depleted of resources and/or contaminated with waste products. While the unstable nature of this upper environment

makes it difficult for a lone individual to safely acquire resources without being drowned, when working together in groups, larvae can collectively dig deep enough to reach these submerged nutrients, and feed relatively safely for longer due to better ventilation. For a cluster to be successful, larvae must rely on their visual, mechanosensory, and gustatory sensory systems (Mast et al., 2014; Dombrovski et al., 2017; Jiang et al., 2020). Larvae must find each other using movement and chemical cues produced by their conspecifics (Mast et al., 2014; Slepian et al., 2015), and must coordinate their contraction movements so that the cluster can maintain its structural integrity (Dombrovski et al., 2017). Vision is of great importance, as blind larvae or larvae reared in constant darkness, engage in less foraging (Dombrovski et al., 2017). There is also a crucial social experience component to a successful cluster – flies that are isolated during their early instar phases are incapable of later joining clusters effectively (Slepian et al., 2015; Dombrovski et al., 2017).

The benefits of this social behaviour have been measured in several studies which have found that when larvae engage in cluster feeding in degraded environments there is higher survivorship and larger adult body sizes (Khodaei & Long, 2020; Dombrovski et al., 2020). At the same time, joining a cluster does entail some risks to the participants. First, there is the potential that the mine may fail due to the presence of incompatible participants who disrupt the synchronous reciprocating digging (Dombrovski et al., 2020), or the mine may collapse due to the liquidity of the environment, possibly leading to suffocation. Even if the groups are successful at reaching unexploited resources, the nutritional rewards are not immediate as they first need to be ‘socially digested’ via the production and excretion of larval digestive enzymes (Gregg et al., 1990; Sakaguchi & Suzuki, 2013). Finally, while cluster feeding does grant access to otherwise inaccessible nutrients, larvae engaged in

cluster feeding may exhibit developmental delays in pupation and eclosion (Dombrovski et al., 2020). The magnitude of these costs and benefits, and thus the expression of clustering behaviour, may be strongly influenced by the larval environment. Cluster feeding is not seen in vials of *D. melanogaster* until the medium has become degraded into a semi-liquified form and the number of clusters and the number of larvae in clusters increases when there is a higher density of larvae present (Dombrovski et al., 2017; Khodaei et al., 2020). This is presumably because clustering does not become a desirable undertaking while the available surface environment still has sufficient resources, low levels of waste products and/or the terrain is sufficiently stable to favour an individual-foraging strategy (Khodaei et al., 2020). Flies kept at low larval densities, where there is little competition for resources, rarely engage in clustering (Khodaei et al., 2020; Dombrovski et al., 2017). Together, this suggests that larvae are sensitive to changes in the nutritional and physical qualities of their environment and may adjust their degree of sociality depending on which strategy yields the greatest net benefit.

In this study, we set out to explore decision-making in groups of *D. melanogaster* larvae by measuring the timing, the frequency, the membership and the size of social feeding clusters in a variety of environments differing in their nutritional qualities and configurations. *D. melanogaster* has the potential to be a powerful model species to test sociobiological hypotheses regarding the factors which mediate social behaviour (Chen & Sokolowski, 2022) and understanding the dynamics of group foraging is of great importance. Although this paper is focussed primarily on fruit fly larvae, cooperative foraging is not constrained to this species, and similar cooperative foraging behaviour has been seen in other non-eusocial insect larvae. For instance, neotropical treehoppers

(*Calloconophora pinguis*), eastern tent caterpillars (*Malacosoma americanum*), and ladybird beetle (*Adalia bipunctata*) larvae use vibrational, secretory, and chemosensory signals (respectively) to engage in cooperative foraging behaviour, predominantly with siblings (Fitzgerald & Peterson, 1988; Hemptinne, et al., 2000; Cocroft, 2005).

The limited knowledge to date about the factors that contribute to the collective foraging behaviour of *D. melanogaster* larvae, makes making clear a priori hypotheses difficult. The current study constitutes one of the first steps in understanding the decisions made by larvae engaging in this cooperative behaviour. We speculated that varying environmental factors would alter the nature of clustering, but we could not predict exactly which changes we would observe. As time progresses, more larvae will reach the third instar stage, so more clustering will occur, similarly, as larvae start to enter the wandering stage and pupate, there will be a decrease in clustering (Khodaei et al., 2020).

Methods

Population history and maintenance

All flies used in this study originated from the large, outbred, wild-type *Ives* (hereafter 'IV') population of *D. melanogaster*, which originated as a sample of wild-caught flies from South Amherst, MA, USA, in 1975, and has been maintained under standardized culture conditions since 1980 (Rose, 1984). Our population lineage was obtained from the Chippindale lab (Queen's University, Kingston, Ontario) in 2011. These flies are normally cultured in vials containing 10ml of standard fly food media (described below). Flies are housed at 25°C and ~60% humidity, on a 12hL: 12hD diurnal light cycle (lights on at 9:00 am), and this population is maintained on a discrete (non-overlapping) 14-day generation culture cycle. At the start of every generation, all eclosed adult flies are

removed from their vials, mixed *en masse* under light CO_2 anesthesia, and transferred to new vials. Flies are left to oviposit in these vials for up to 20h, at which time adults are removed and the number of eggs in each vial is standardized to a density of 100 per vial (Tennant et al., 2014)

General experimental protocol

To test if larval clustering dynamics were influenced by environmental resource availability, we created experimental vials in which we manipulated the type and/or distribution of nutritional resources. Our experiments used two types of fly food media whose recipes differed in their overall nutritional content. For our nutrient-rich food, we used our standard fly food (Rose, 1984), which contains 120.6g/L peeled banana, 30.2g agar/L, 15.1ml/L each of dark and light corn syrup, 22.1ml/L of barley malt, 32.2g/L of (cooked) active dry yeast, 42.4ml/L of 95% anhydrous ethanol and 2.1g/L of p-hydrobenzoic acid (*aka* 'tegosept'). For our nutrient-poor food we modified the standard food recipe by reducing the concentration of all ingredients by 50% (except for the non-nutritive ingredients agar and tegosept). For the benefit of the observer we added different food-grade dyes (Club House Brand, McCormick & Company, Inc.) to the media so that they could be distinguished from each other (the observer was blind to the color-coded identity of the nutritional quality of the foods).

In our first experiment, we created three different conditions, each representing a distinctive nutritional environment. For each condition we created vials containing 10 ml of media, and 100 newly-laid fruit fly eggs. For the high nutrition condition, we used our standard food, and for the low nutrition condition we used the nutrient-poor media. In preparing vials for the third "50:50" condition, we first placed a thin polystyrene strip down

the center of each vial and added 5 ml of media to each side of the partition (Figure S2). Once the food was cooled, the barrier strip was removed, thus allowing larvae free access to both types of media and eliminating any edge effects across the two media. A line delineating the division in the substrates was marked on the outside of the vial with a permanent marker for future reference. In this experiment we established 38 replicate vials per condition.

For our second experiment we created four different conditions, each representing a unique combination of nutritional environments. For each condition we created vials containing 10 ml of media, and 100 newly-laid fruit fly eggs. We prepared vials by first adding 8ml of food to a vial, chilling it sufficiently to harden and then adding 2ml of food on top (Figure S3). We created all possible combinations of stratified vials using the standard and the low nutrient food: 38 replicate vials of (upper: lower) standard: low food; 40 replicate vials with low: standard food; 13 replicate vials with standard: standard food; and 14 replicate vials with low: low food. We created fewer replicate vials with the same upper and lower food types than mixed food types as these combinations (standard: standard and low: low) are functionally the same as the standard and low nutritional quality condition vials in the first experiment. We used different colours and shades of dye for the media used in for upper and lower layers so that even when they were of the same nutritional quality, the boundary between layers could be seen (at least initially).

The larvae used in both these experiments were obtained by first transferring adult IV flies into oviposition chambers constructed from half-pint polypropylene bottles (Genesee Scientific; 32-130) whose openings were covered with petri dishes (35mm diameter; Nunc, 12-565-90) containing grape juice / agar medium (Sullivan et al., 2000) for

ovipositing. The next morning (~18h later) we carefully collected sets of 100 eggs and transferred them to the experimental vials. For both experiments, vials were initially incubated at the lab for 48h at which time they were carefully transferred to a portable incubator (DT2-MP-47L DigiTherm Heating/Cooling Incubator, Tritech Research, Inc.) located in the corresponding author's residence for the experiment's observation phase (due to our institute's COVID-19 research activity restrictions).

Vials were individually labeled and were observed three times per day for signs of clustering, in sessions spaced three hours apart (~10:00 am, ~1:00 pm, ~4:00 pm). The order in which vials were observed in each session was randomized. At the beginning of each day (9:00 am), vials were removed from the portable incubator and allowed to acclimate to the ambient conditions of the room for ~1h. Each vial was then inspected for the presence of clusters which was defined as a grouping of 2 or more larvae which were all feeding in a downward direction creating a depression in the food media (*similar to* Khodaei & Long, 2019). If any clusters were found, the vial was inserted into a lightbox, adjacent to a scale and each cluster was photographed using a Canon EOS 100D camera mounted on a tripod. Feeding clusters were defined as being distinct from one another if there was evidence that larvae in the two groups were not working together (*i.e.* oriented in opposite directions) and separated by at least 0.5 mm between the two groups. Once all clusters had been photographed, the vials were returned to their respective racks. After the completion of the third session of photos, vials were placed back into the incubator. The process continued daily until no clusters could be observed (~ the 7^{th} day post-vial establishment). Photos were then inspected for presence of clustering, where clustering had to have occurred below the surface level to differentiate clustering from foraging at the

surface. We counted the number of visible larvae which were within the contour of the feeding cluster, and the total number of clusters present in a vial. For vials in experiment 1's 50:50 condition, clusters were classified as being on the standard side, the low nutrition side, or on the border (if the cluster equally straddled both types of media). From each image we measured the depth (the distance from the food surface level to the lowest point in the depression) and width (the distance from one side of the depression to the other at the food surface level) of each cluster using ImageJ software (v1.53e, Schneider et al., 2012) to the closest pixel. A scale bar was used to convert pixels to cm (rounded to 3 decimal places), using a sample image from each session. Due to technical issues, photos from day 4, session 3 were lost.

Statistical Analyses

Due to low overall numbers of clusters that were observed on days 1, 2, and 7 in Experiment 1, as well as on days 1 and 8 in Experiment 2, data from those dates were excluded from analyses. We used R (v.3.6.1, R Core Team, 2019, https://r-project.org) and JASP (v.0.16, JASP Team, 2021, https://jasp-stats.org) to conduct the analyses and visualize the results. For both the cluster depth and width data, separate linear mixed models (LMM) were created with day and condition as fixed factors, and vial and session included as random factors, a random-intercept and random-intercept and slope model was created to determine which best fit the data. For the number of individuals in a cluster generalized linear mixed models (GLMM) were created using functions from the from the *lme4* library (Bates et al., 2005) with Poisson error distributions. We assumed each session was independent as 3 hours has been shown to be sufficient time for clusters to disintegrate (TAFL, *pers obs*.). As we included zero as an option for the number of clusters per vial per

condition, we used a zero-inflated regression model to account for the overabundance of zeros. All permutations of zero-inflated (ZI) and zero-altered (ZA), Poisson and Negative Binomial models were created, along with all permutations of interactions or lack thereof. Akaike Information Criterion (AIC) values were used for model selection and are listed in Table S1. A contingency table was created to identify whether in Experiment 1 the number of clusters differed between the two sides of the 50:50 condition vials across days.

Results

Experiment 1

Number of clusters: There was a significant main effect of condition and day, along with the interaction on the number of clusters observed (condition: χ^2_2 =11.30, p = 0.004; day: χ^2_1 = 13.25, p = 0.0003; interaction: χ^2_2 = 7.79, p = 0.02; Figure 1A,1B). Both the standard and 50:50 conditions decreased in number of clusters, with the low condition increasing in clusters over time (Figure 1A, 1B).

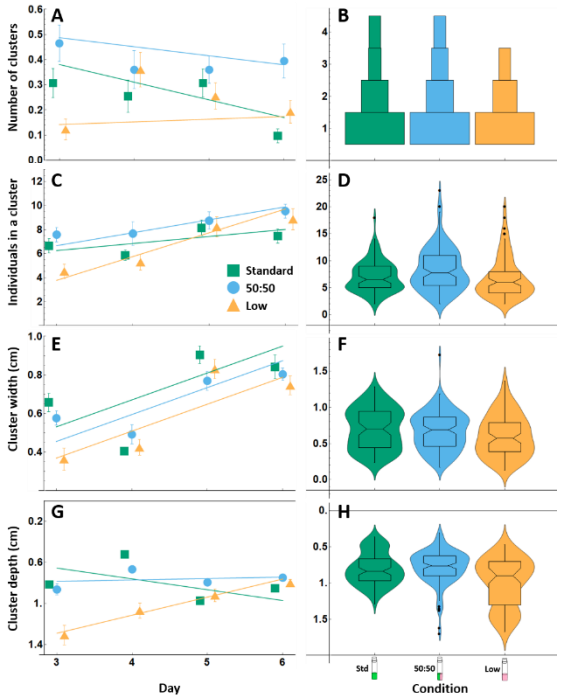

Figure 1: Visualization of the temporal patterns (**A**, **C**, **E**, **G**) and overall distributions by conditions (**B**, **D**, **F**, **H**) for larval clusters measured in Experiment 1. **A, B:** mean number of clusters counted in vials; **C**, **D:** mean number of individuals within each cluster; **E**, **F**: mean width of clusters; **G**, **H**: mean depth of clusters. Panels **A**, **C**, **E**, **G** show regression lines superimposed on raw data for clusters measured in conditions where vials contained standard media (green squares), low nutritional media (orange triangles) or a 50:50 mix of media (blue circles). Panels **B**, **D**, **F**, **H** show violin plots of the data for each treatment across all time points, and boxplots in which the boxes contain the middle 50% of the data (inter-quartile range, IQR), with the location of the medians indicated by solid bars inside the boxes. Values > ± 1.5 IQR outside the boxes are designated as outliers and marked as circles.

Individuals within clusters: Day had a significant effect ($\chi^2_1 = 61.72$, $p < 0.001$; post-hoc tables are in Table S2; Figure 1C) on the number of individuals in a cluster, and there was a significant interaction between the two main factors ($\chi^2_2 = 16.48$, p = 0 .0003; Figure 1C).There was an effect of condition ($\chi^2_2 = 11.59$, p = 0.003; Figure 1D) on individuals within a cluster, with significantly more individuals in the 50:50 than the low condition (post-hocs: p = 0.008; see Table S2).

Cluster structure: There was a significant effect of day ($F_{1,292} = 116.05$, $p < 0.001$; Figure 1E), condition ($F_{2,100} = 6.52$, p = 0.002; Figure 1F; post-hocs can be found in Table S3), and on cluster width. The clusters in the low condition vials were, on average, significantly narrower than those in standard condition vials (post-hoc: p <0.001). Cluster width increased over time in all conditions, with the interaction most visible between the low and 50:50 conditions (Figure 1E).

There was no significant main effect of day ($F_{1,290} = 3.77$, p = 0.053; Figure 1G, post-hocs can be found in Table S4), but there was a significant main effect of condition ($F_{2,100} = 9.93$, p = 0.0001; Figure 1H), which should be interpreted with caution due to the significant interaction between condition and day ($F_{2,290} = 21.75$, $p < 0.0001$, Figure 1G). Between conditions, the low food quality vials had significantly deeper clusters (post hocs: $p < 0.001$ for comparing low to standard, and 50:50, respectively; Figure 1H). While mean cluster depth was fairly stable over time in both the standard and the 50:50 condition vials, in the low nutrition trials, mean cluster depth decreased over time (Figure 1G).

Larval choice analysis: One of the main rationales for Experiment 1 was to investigate how the quality of food affects larvae's choice of where and how to cluster. For vials in the 50:50 condition, the food substrate was vertically transected, with one side at

standard nutrition, and the other with poor nutrition, which allowed us to test whether larvae actively bias where to dig. After removing clusters located on the border (as no clear "choice" was made), a contingency analysis revealed there was a significant association between day and the side of the cluster ($\chi^2_4 = 36.91$, $p < 0.001$; Figure 2). While the number of clusters on the standard side was initially greater than expected by chance, this trend had reversed by day 5, after which the clusters on the low nutrition side were more numerous than expected (Figure 2).

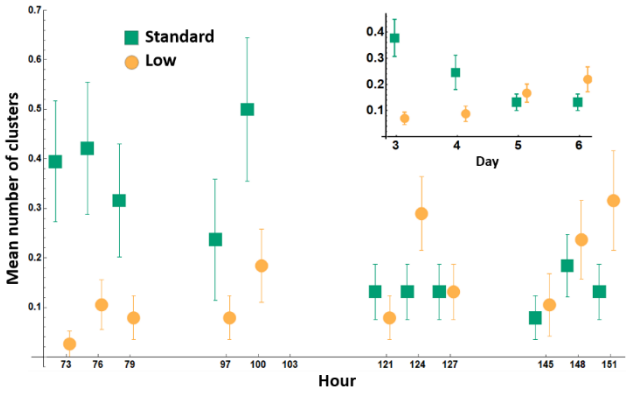

Figure 2: Temporal patterns of the mean number of clusters and location of larval clusters observed in the 50:50 condition vials in Experiment 1. While the number of clusters on the standard side (green) was initially high, their abundance decreased over time; the number on the low side (orange) increased later in the study. The inset shows the mean number of clusters by day rather than hour.

Experiment 2

Number of clusters: There was a significant effect of day ($\chi^2_1 = 15.12$, $p = 0.0001$; Figure 3A), and condition on the number of clusters ($\chi^2_3 = 16.52$, $p = 0.00089$; Figure 3B). As time passed, the number of clusters for low:low and std:low increased, whereas the number stayed generally consistent for conditions low:std and std:std (Figure 3A,3B).

Individuals within clusters: There were significant effects of day ($\chi^2_1 = 42.28$, $p < 0.001$; Figure 3C; post-hocs can be found in Table S5) and condition ($\chi^2_3 = 14.88$, $p = 0.002$; Figure 3D). The mean number of individuals in a cluster increased over time. There were significantly more individuals clustering in the standard: low condition compared to all other conditions (post hoc: $p < 0.001$ for all comparisons see Table S5).

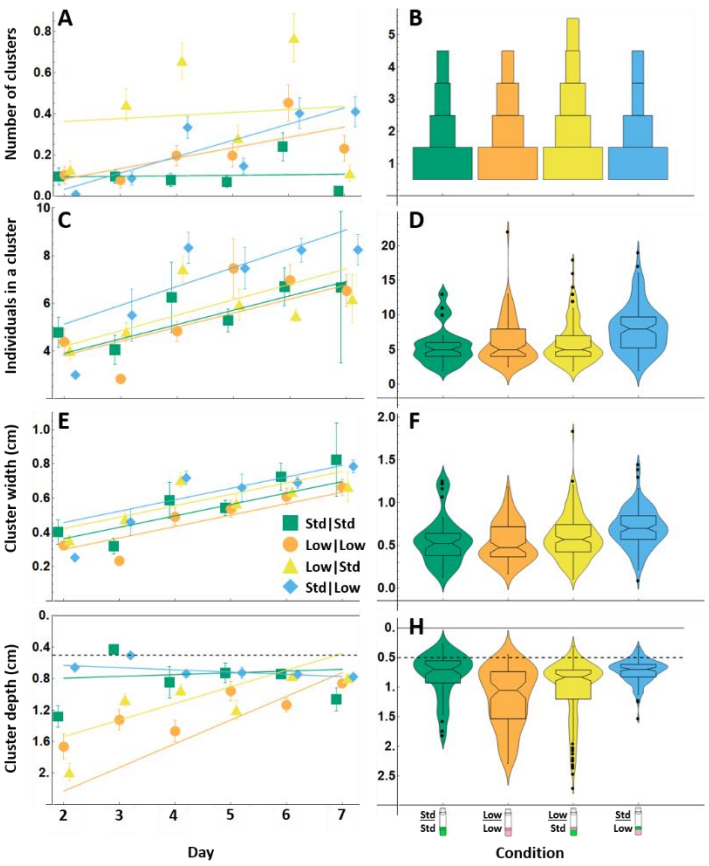

Figure 3: Visualization of temporal patterns (**A**, **C**, **E**, **G**) and overall condition distributions (**B**, **D**, **F**, **H**) for clusters measured in Experiment 2. **A**, **B**: mean number of clusters counted in each vial; **C, D**: mean number of individuals within each cluster; **E**, **F**: mean width of clusters; **G**, **H**: mean depth of clusters. Panels **A**, **C**, **E**, **G** have regression lines superimposed on the data for clusters measured in conditions where vials contained standard media (green squares), low nutritional media (orange circles) or standard media on top with low media on the bottom (blue diamonds), or low media on top with standard media on the bottom (yellow triangles). Panels **B**, **D**, **F**, **H** show violin plots of the data from each treatment across all time points, and boxplots in which the boxes contain the middle 50% of the data (inter-quartile range, IQR), with the location of the medians indicated by solid bars inside the boxes. Values > ± 1.5 IQR outside the boxes are designated as outliers and marked as circles. Boxplot whiskers extend to the min/max values that are not outliers. Dashed horizontal lines in **G** and **H** indicate the depth (0.5 cm) of the initial boundary between the two media types.

Cluster structure: The interaction between day and condition was removed as the AIC was similar to that of the model that included it but was not significantly different. No other deletions of fixed effects decreased the AIC. There were significant main effects of day ($F_{1,557} = 71.93$, $p < 0.001$; Figure 3E; post-hocs can be found in Table S6) and condition ($F_{3,97} = 5.22$, $p = 0.002$; Figure 3F). The low: low condition had significantly narrower clusters compared to the low: standard ($p = 0.036$) and the standard: low ($p < 0.001$) conditions. The low: standard condition had significantly narrower clusters compared to standard: low ($p = 0.002$). The standard: low condition had significantly wider clusters compared to standard: standard ($p < 0.001$; Figure 3F).

There was a significant interaction between day and condition

($F_{3,554}$ = 18.86, $p < 0.0001$). There were also significant main effects of day ($F_{1,554}$ =64.29, $p < 0.0001$; Figure 3G; post-hocs can be found in Table S7) and condition ($F_{3,97}$ = 6.77, $p < 0.0001$; Figure 3H). The low: low condition had significantly deeper clusters compared to all other conditions ($p = 0.001$ low: low to low: standard; $p < 0.001$ for all other comparisons). The low: standard condition had significantly deeper clusters than standard: low and standard: standard ($p < 0.001$; $p = 0.003$; respectively).

Discussion

The formation of feeding clusters among groups of third-instar *D. melanogaster* larvae presents an excellent opportunity to study the ecological, evolutionary, and neurogenetic factors that may facilitate or impede social interactions (Durisko et al. 2014, Dombrovski et al., 2017; Khodaei & Long, 2019; Chen & Sokolowski, 2022). However, much remains unknown about how the physical and nutritional environments influence the expression of cluster feeding. In this study, we set out to explore plasticity in this important social behaviour across nutritional environments. The third instar is a crucial developmental stage for *D. melanogaster* larvae, whose ability to survive and undergo metamorphosis depends on their ability to consume nutrients (Sang, 1956; Boggs, 1981; Prasad et al., 2003). Clustering in *D. melanogaster* generally occurs when the surface environment is sufficiently soft (Durisko et al., 2014), as well as when competition for resources is high, such as at higher population densities,or when food quality is poor (Dombrovski et al., 2017, Khodaei et al., 2020). We systematically manipulated food quality and distribution to investigate the potential for flexibility in expression of this complex social behaviour. Given what is known about sociability in larvae (Durisko & Dukas, 2013; Slepian et al., 2015; Dombrovski et al., 2017), we suspected that they may actively assess their

environment and collectively decide to take advantage of potential resources in locations that yielded the greatest nutritional benefits. Our experiments revealed that larval feeding cluster abundance, membership, and morphology are dynamic and vary in ways which suggests that larvae are responsive to the specific conditions present in their local environment.

Across both our experiments, the number of clusters, and the participation in cluster feeding generally increased over the course of the assay. This was an expected pattern, as the number of individuals in the cohort reaching the third instar threshold within each of the vials increases with time, and concurrently the nutritional quality and physical stability of the environment progressively deteriorates (Khodaei et al., 2020). Together, these conditions lead to increasing need, opportunities, and potential benefits of clustering compared to a solitary foraging strategy. Overall, the mean cluster width increased and the mean cluster depth decreased over time in our two sets of experiments, results which should be interpreted in the context of the structural integrity of the media in the early compared to the later days of the assays (Kim et al., 2017; Durisko et al., 2014). Clusters that form in the first few days of the experiment are established in media that can physically support deeper clusters, due to the relative rigidity of the surface-level environment. However, as time passes the cumulative effects of larval foraging, natural decomposition, and the digestive effects of secreted enzymes, makes the surface environment increasingly liquefied. In such unstable environments deeper clusters are likely harder to dig and more prone to cave-ins. By widening the entrance to the mine, the foraging group potentially decreases the likelihood of collapse, and requires a lower level of energetic investment by individual larvae.

Changes in environmental nutritional availability were associated with different cluster characteristics. In Experiment 1, larvae in the low nutrition condition initially formed narrow and deep clusters, whereas groups of larvae under standard conditions formed wider and shallower mines. These differences reflect the behavioural plasticity of the larvae in response to nutritional availability. While larvae in the standard nutrition condition vials may have been able to acquire sufficient resources closer to the surface, those in the low nutrition vials likely dug deeper mines in search of potentially better quality resources – in both richness and structural integrity – which were lacking near the surface. This sensitivity to resource availability was also seen in the 50:50 condition vials, where there was a gradual change in the frequency of clusters from the standard side to the low nutrition side. This adjustment likely occurred as the absolute and relative quality of the standard side media decreased over time, though the structural integrity of the media on the low side was likely higher than the clustered-upon standard side for much of the assay. These results suggest that there are active trade-offs between the benefits of better food produced by deeper mines and the energetic costs of producing and maintaining them without collapse. Interestingly, the 50:50 condition vials had the most individuals in each cluster, compared to the standard and low condition vials, but we found no significant effect of time or resource distribution on the number of clusters. This means that, despite differences and changes in food quality, larvae did not alter the number of clusters they formed, but rather altered the participation in clusters. Whether this is a consequence of the space constraints in our vials limiting the total number of distinct clusters possible is unknown, and future studies could explore the composition and frequency of clusters over larger surfaces.

In Experiment 2, the highest mean number of individuals within a cluster was found in the standard: low condition, followed by the low: low condition, the low: standard condition, and the standard: standard condition (upper layer: lower layer, respectively). The number of individuals within a cluster appears to be more strongly dependent on the quality of the media below the surface than at the surface itself, with more larvae collectively clustering when there are fewer rewards in the buried layers. Mean cluster width increased over time in all conditions, with standard: low condition vials having on average the widest clusters, followed by the low: standard condition vials, the standard: standard condition vials, and finally the low: low condition vials. This pattern is especially interesting when coupled with the observations that larvae in the low: low condition vials had the deepest clusters, followed by the low: standard condition vials, standard: standard condition vials, and the standard: low condition vials. This means that for the standard: low condition, larvae were able to assess the difference in food quality across the layers and alter their cluster structure to create wider and shallower clusters, taking advantage of the better quality of food closer to the surface, while larvae in the low: low vials, conversely, dug deeper and narrower mines likely in the pursuit of nutrients located at lower levels. Larvae in the low: standard conditions formed clusters that were both fairly wide and deep, allowing for greater access to the better resources located beneath the surface. Finally, clusters in the standard: standard condition vials were relatively thin and shallow, likely as they do not experience the same degree of resource scarcity experienced in the other conditions, and consequently engage in less extensive cluster feeding. These observations, along with the results of the first experiment, strongly indicate that clustering third instar *D. melanogaster* larvae are continuously taking in information about their environment and

adjusting their social behaviours accordingly, presumably in a way that balances the costs and risks against the benefits of clustering.

The successful establishment of feeding clusters requires coordination, cooperation, and prior sensory experience obtained during a short, discrete amount of time (Mast et al., 2014; Slepian et al., 2015; Dombrovski et al., 2017; Wong et al., 2017; Jiang et al., 2020). These factors can be individually isolated and examined under the controlled environments in which *D. melanogaster* can be housed and using the extensive range of genetic tools that have been developed for this model species (Ashburner et al. 2005; Greenspan, 2004; Zhang et al., 2010). Clustering in *D. melanogaster* may prove to be an ideal system in which to study the mechanisms that underlie the origins of social behaviour (Chen & Sokolowski, 2022). Our exploration of the effect of variation in food quality is only one of many potential factors that can affect clustering behaviour, and future studies may focus on other avenues to further manipulate cluster structure to understand the bounds of its flexibility. For example, previous research has suggested that the rigidity of the media affects the likelihood to cluster, where too soft a medium made moving difficult, and too hard a medium made digging difficult (Durisko et al., 2014; Kim et al., 2017). Coupled with experimentally changing the food quality, simultaneously altering the initial firmness of the environment may result in different patterns of cluster formation as the costs and benefits of clustering are changed. Additional insight into the economics of social foraging might be obtained by changing the abundance of carbohydrates and/or proteins in the media, as these play an important role in the development of larvae and their future success during adulthood (e.g. Rodrigues et al., 2015), and their relative availability may promote or prevent clustering. The impact of maternal behaviour on larval social behaviour is

another element worthy of more scrutiny. In *D. melanogaster*, females choose where to oviposit their eggs, and their decision may result in them laying their eggs closer to other groups, thus increasing the subsequent larval density in that area (Zhang, et al. 2020; Churchill et al., 2021). The effects of being raised in an environment with poor nutrition on the propensity of future generations to cluster can aid in understanding if there is transmission of this social behaviour between generations. Future studies could also investigate if there are differences in the characteristics of individuals that enter clusters forming at different days. Such a study may reveal consistent individual differences in social foraging behavioural tendencies and whether some larvae are more likely to dig deeper, which is more beneficial before the environment undergoes extensive liquefication. There is an extensive literature on individual differences in *D. melanogaster* flies and larvae (*e.g.* Mery et al., 2007; Reaume & Sokolowski, 2009; Alwash et al., 2021), though it is still being investigated if and how these differences translate into behaviours within the same cluster. Outside of fruit flies, similar questions can be asked about other insects that engage in cooperative behaviour, and could be used to examine how the use of different sensory modalities affects the flexibility of their behaviours. For example, treehoppers use vibrational signals to communicate to others a new source of food; how this signal changes in accordance with a changing environment and the resulting flexibility of group behaviours is unclear (Cocroft, 2005).

In summary, by systematically manipulating the quality and distribution of the substrate on which groups of third instar *D. melanogaster* larvae foraged, we demonstrate that larvae are flexible decision-makers who alter their propensity to cluster and the configuration of the clusters they form, depending (at least partially) on the quality of food

they encounter at the surface and the spatial distribution of food both vertically and horizontally. It is likely that many other environmental and internal factors shape larval behavioural decisions about collective foraging, and exploring these will improve our basic understanding of how cognitive processes contribute to collective behaviours both in this emerging model, as well as in other social species.

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Supplementary Information

Figure S1. Illustration of a Drosophila melanogaster larval feeding cluster with lines illustrating how width and depth of each cluster were measured. The width of each cluster was defined as the horizontal distance between the outermost cluster group members, while the depth of each cluster was defined as the vertical distance from the media surface level to the lowest point in the foraging group. We also counted the number of larvae along the contour of the feeding cluster that were visible through the transparent wall of the vial as an index of how many larvae were co-operating.

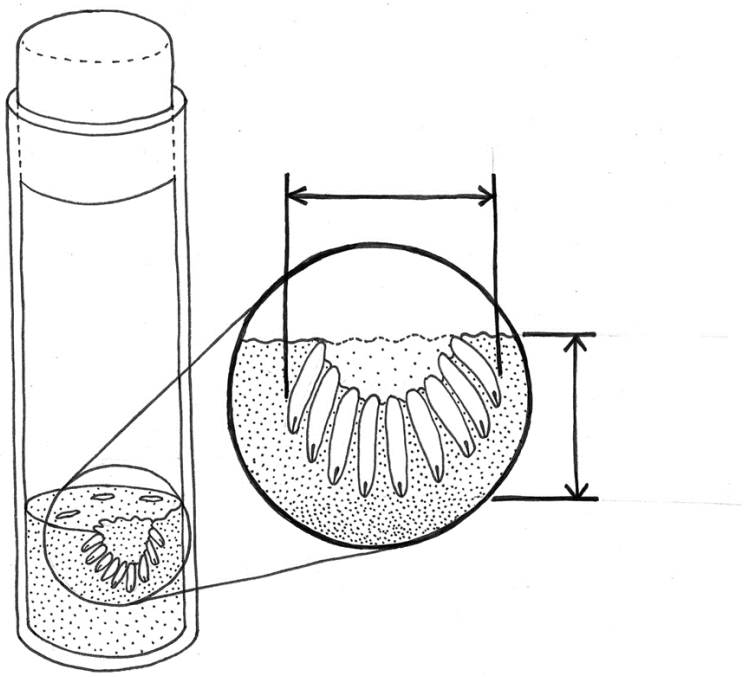

Figure S2. Photo illustrating the split media condition in Experiment 1. Each side of the vial contained 5ml of media. A “raft” of eggs has been placed at the mid-line in the left photo. The observer was blind to the identity of the low and standard media.

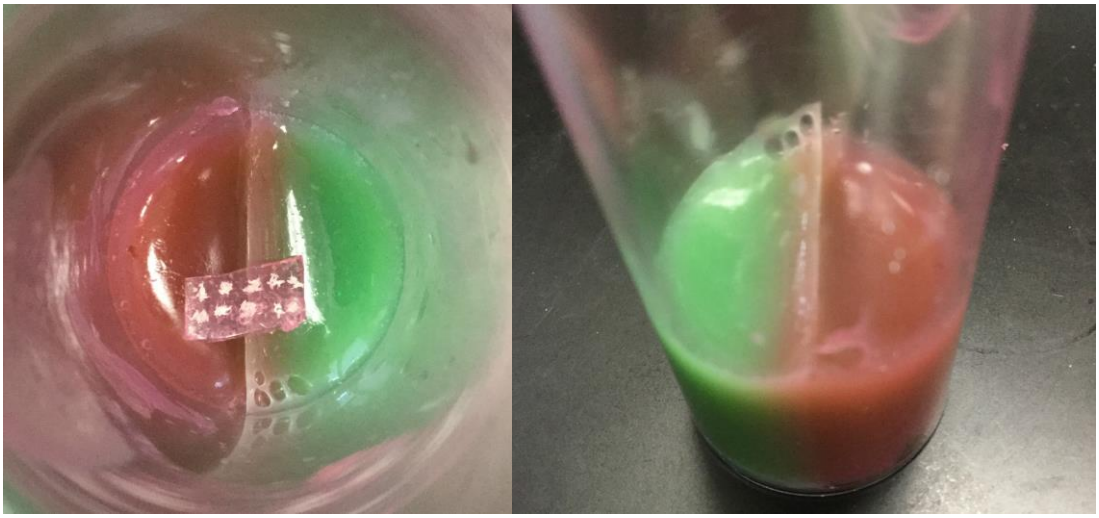

Figure S3. Photo illustrating the different stratification vials. In each vial, 8ml of media was poured, then cooled. An additional 2ml of media was then added on top. In this way, we could create environments with (left to right) low nutritional media on top of standard media; standard media on top of low media; standard media on top of standard media; low media on top of low media. The observer was blind to the specific combination of media present in any vial.

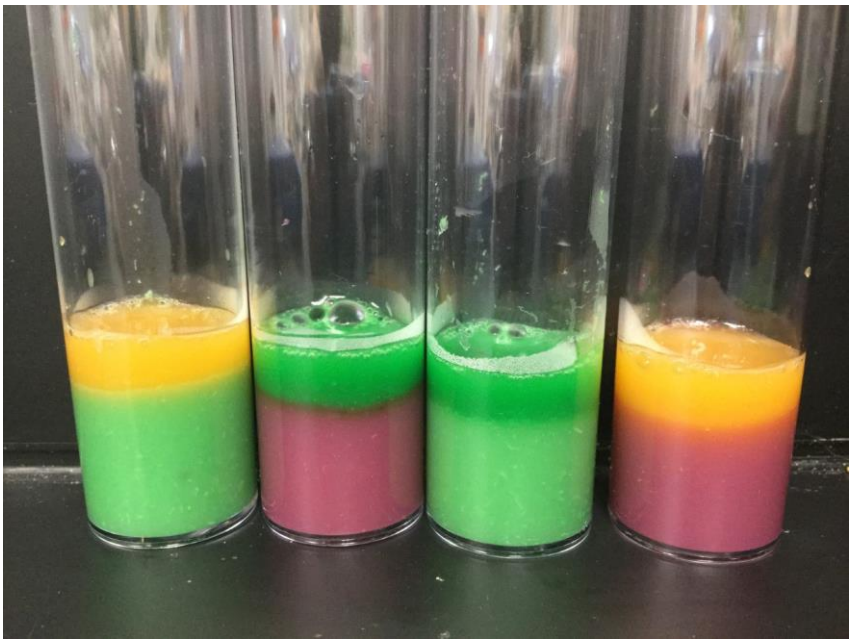

Table S1. Models tested and their respective AIC values for Experiment 1 and 2. AIC values for the variety of regression models tested for Experiment 1 and 2 data. All fixed factors were Day and Condition. *** p – value comparing models is < .0001. Where AIC values were not significantly different, the simpler model was chosen.

Dependent variables	Random factors	Model type	AIC values
Experiment 1			
Number of clusters (count)		Poisson	1846.90
		Negative Binomial	1804.2
		Zero-inflated Poisson	1789.42
		Zero-altered Poisson	1802.18
		Zero-inflated Negative Binomial	1791.42
		Zero-altered Negative Binomial	1804.18
Number of individuals in a cluster (count)	Day | vial	Random-intercept and slope	2154.5
	Vial	Random-intercept	2151.8***
	Day | vial	No interaction	2164.2***
Depth (cm)	Vial	Linear model	135.82***
	Session | vial	Random-intercept and slope	69.02
	Vial	Random-intercept	103.53***
Width (cm)	N/A	Linear model	97.72***
	Session | vial	Random-intercept and slope	84.99
	Vial	Random-intercept	80.99***
Experiment 2			
Number of clusters (count)		Poisson	2984.33
		Negative Binomial	2794.00
		Zero-inflated Poisson	2755.49
		Zero-altered Poisson	2764.98
		Zero-inflated Negative Binomial	2757.49
		Zero-altered Negative Binomial	2766.98
		Zero-inflated Poisson, no interaction of fixed terms	2754.56
Number of individuals in a cluster (count)	Day | vial	Random-intercept and slope	3300.2
	Vial	Random-intercept	3276.6
	Day | vial	No interaction	3277.1
Depth (cm)	N/A	Linear model	702.19***
	Session | vial	Random-intercept and slope	554.69***

	Vial	Random-intercept	595.61***
Width (cm)	N/A	Linear model	125.06***
	Session | vial	Random-intercept and slope	113.56***
	Vial	Random-intercept	109.56***

Table S2. Post-hoc tests with Tukey correction, to compare each day and condition, respectively, to individuals within a cluster in Experiment 1.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	0.598	0.524	1.143	0.663	
	5	-1.996	0.531	-3.760	0.001	**
	6	-2.548	0.574	-4.438	< .001	***
4	5	-2.594	0.520	-4.991	< .001	***
	6	-3.147	0.564	-5.578	< .001	***
5	6	-0.553	0.571	-0.969	0.767	

** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 4

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
A	AB	-0.917	0.495	-1.854	0.154	
	B	0.542	0.567	0.956	0.605	
AB	B	1.460	0.489	2.988	0.008	**

* p < .05, ** p < .01

Note. P-value adjusted for comparing a family of 3

Note. Results are averaged over the levels of: Day

Table S3. Post-hoc tests with Tukey correction, to compare each day and condition, respectively, to width in Experiment 1.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	0.115	0.033	3.512	0.003	**
	5	-0.295	0.033	-8.887	< .001	***
	6	-0.247	0.036	-6.885	< .001	***
4	5	-0.410	0.033	-12.614	< .001	***
	6	-0.363	0.035	-10.269	< .001	***
5	6	0.048	0.036	1.337	0.540	

** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 4

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
A	AB	0.068	0.030	2.241	0.066	
	B	0.126	0.035	3.621	< .001	***
AB	B	0.058	0.030	1.936	0.130	

** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 3

Note. Results are averaged over the levels of: Day

Table S4. Post-hoc tests with Tukey correction, to compare each day and condition, respectively, to depths of clusters in Experiment 1.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	0.230	0.038	6.010	< .001	***
	5	0.097	0.039	2.514	0.059	
	6	0.187	0.045	4.174	< .001	***
4	5	-0.133	0.035	-3.776	0.001	**
	6	-0.043	0.042	-1.023	0.736	
5	6	0.090	0.042	2.130	0.145	

* p < .05, ** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 4

Note. Results are averaged over the levels of: Condition

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
A	AB	0.013	0.033	0.379	0.924	
	B	-0.225	0.038	-5.886	< .001	***
AB	B	-0.237	0.033	-7.220	< .001	***

*** p < .001

Note. P-value adjusted for comparing a family of 3

Note. Results are averaged over the levels of: Day

Table S5. Post-hoc tests with Tukey correction, to compare each day and condition to individuals within a cluster in Experiment 2.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	-0.395	0.629	-0.628	0.989	
	5	-2.866	0.582	-4.924	< .001	***
	6	-2.179	0.630	-3.458	0.008	**
	7	-1.933	0.562	-3.438	0.008	**
	8	-2.659	0.619	-4.297	< .001	***
4	5	-2.471	0.445	-5.551	< .001	***
	6	-1.785	0.507	-3.522	0.006	**
	7	-1.538	0.419	-3.672	0.004	**
	8	-2.264	0.492	-4.599	< .001	***
5	6	0.687	0.447	1.537	0.640	
	7	0.933	0.344	2.709	0.075	
	8	0.207	0.431	0.481	0.997	
6	7	0.246	0.421	0.585	0.992	
	8	-0.480	0.494	-0.971	0.927	
7	8	-0.726	0.404	-1.799	0.467	

* p < .05, ** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 6

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
bb	bg	0.031	0.330	0.095	1.000	
	gb	-1.859	0.369	-5.035	< .001	***
	agg	0.471	0.471	1.000	0.749	
bg	gb	-1.890	0.320	-5.909	< .001	***
	agg	0.439	0.433	1.015	0.741	
gb	agg	2.329	0.463	5.026	< .001	***

*** p < .001

Note. P-value adjusted for comparing a family of 4

Table S6. Post-hoc tests with Tukey correction, to compare each day to widths of clusters in Experiment 2.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	-0.098	0.049	-1.991	0.349	
	5	-0.323	0.045	-7.108	< .001	***
	6	-0.219	0.049	-4.462	< .001	***
	7	-0.271	0.044	-6.184	< .001	***
	8	-0.355	0.048	-7.349	< .001	***
4	5	-0.225	0.035	-6.481	< .001	***
	6	-0.122	0.039	-3.080	0.026	*
	7	-0.173	0.033	-5.311	< .001	***
	8	-0.257	0.038	-6.694	< .001	***
5	6	0.103	0.035	2.965	0.037	*
	7	0.051	0.027	1.917	0.392	
	8	-0.032	0.034	-0.954	0.932	
6	7	-0.052	0.033	-1.580	0.612	
	8	-0.135	0.039	-3.514	0.006	**
7	8	-0.084	0.031	-2.654	0.086	

Note. P-value adjusted for comparing a family of 6

* p < .05, ** p < .01, *** p < .001

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
bb	bg	-0.072	0.027	-2.695	0.036	*
	gb	-0.165	0.030	-5.498	< .001	***
	agg	-0.017	0.038	-0.443	0.971	
bg	gb	-0.093	0.026	-3.563	0.002	**
	agg	0.055	0.035	1.573	0.395	
gb	agg	0.148	0.038	3.928	< .001	***

* p < .05, ** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 4

Table S7. Post-hoc tests with Tukey correction, to compare day and condition to depth of clusters in Experiment 2.

Post Hoc Comparisons - Day

		Mean Difference	SE	t	p_{tukey}	
3	4	0.705	0.080	8.840	< .001	***
	5	0.676	0.074	9.162	< .001	***
	6	0.641	0.080	8.018	< .001	***
	7	0.758	0.071	10.636	< .001	***
	8	0.810	0.078	10.320	< .001	***
4	5	-0.029	0.056	-0.512	0.996	
	6	-0.064	0.064	-1.001	0.918	
	7	0.053	0.053	1.000	0.918	
	8	0.105	0.062	1.679	0.546	
5	6	-0.035	0.057	-0.624	0.989	
	7	0.082	0.044	1.879	0.416	
	8	0.134	0.055	2.448	0.141	
6	7	0.117	0.053	2.201	0.239	
	8	0.169	0.063	2.700	0.077	
7	8	0.052	0.051	1.010	0.915	

*** p < .001

Note. P-value adjusted for comparing a family of 6

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	
bb	bg	0.161	0.043	3.767	0.001	**
	gb	0.406	0.048	8.481	< .001	***
	agg	0.359	0.061	5.884	< .001	***
bg	gb	0.245	0.042	5.900	< .001	***
	agg	0.198	0.056	3.523	0.003	**
gb	agg	-0.047	0.060	-0.780	0.863	

** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 4