

## 6. Microanalysis of *Drosophila* Courtship Behaviour

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**Abstract.** We investigated the temporal structures of behavioural interactions. Our approach is characterized by two major features: data were collected at high temporal and spatial resolutions that revealed acts invisible to the unaided eye and patterns were detected in the collected data using THEME. We investigated courtship displayed by a *fruitless* mutant (*fru*<sup>1</sup>) male, a courtship mutant of *Drosophila melanogaster*, towards mature males and virgin females compared with wild-type males. Novel elements of behaviour revealed at high temporal and spatial resolution were used to augment the repertoire of elements. We showed that frequency of an element was not a reliable indicator of its involvement in patterns. Our findings reveal behaviour structures not apparent to the unaided observer: that *fru*<sup>1</sup> males interact little with their courtrees when courting both wild-type females and males; wild-type females contribute more elements to the common pattern when being courted by *fru*<sup>1</sup> males; wild-type males interact more with females during courtship. The contribution of elements made by wild-type female to the common pattern does not change whether the partner is *fru*<sup>1</sup> male or wild-type male. The co-occurrence of specific pairs of elements, one from each partner, hints at a putative syntax of communication underlying these interactions.

**Keywords:** *Drosophila melanogaster*; courtship behaviour; behavioural patterns; video-recording.

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## 6.1 Introduction

We are interested in how genes control behaviour. For this, we study courtship behaviour of the fruitfly *Drosophila melanogaster* in which it is a well established social interaction behaviour paradigm. This fruitfly also has the best developed molecular genetics of any metazoan. The behaviour of males during courtship has been characterized as consisting of eight steps (orientation following, tapping, wing extension, circling, licking, attempted copulation, copulation) but not much is known about the females [1, 2]. The fruitfly is relatively easy to maintain and has a short generation time. Although it has a simple nervous system (approx.  $10^5$  neurons) and small genome (4 pairs of chromosomes), the fruitfly is able to display complex behaviours [3].

The measure of courtship has been the courtship index (CI) defined as the fraction of the courtship period during which a courter performed courtship towards the courtee. Thus, only a number was used to describe the whole observation period. To augment the presentation of behaviour, another parameter the wing extension index (WEI) was introduced to be used in addition to CI. These two numbers were used to describe the entire observation [1,2, and 4]. There have been efforts to fit the kinetics of mating to second order reaction kinetics [5] and others have applied sophisticated methods like multivariate courtship profile [6]. Whereas these methods summarize and clarify the given data they impose rather than reveal intrinsic structures in the behaviour.

Behaviour is inherently a multidimensional and fraught with nonlinearities: even small changes that may be statistically insignificant could result in massive changes in phenomena. This renders classical statistics methods effective in revealing some salient features. In this chapter, we collected data at high temporal and spatial resolution to capture micro-scale activity observable during courtship behaviour by male carrying a mutant allele of the *fruitless* gene (*fru*<sup>1</sup>). Males carrying this *fruitless* allele court females and also display aberrant behaviour by courting mature males [7-9]. The THEME program was used to detect hidden structures from courtship behaviour data [10, 11]. We investigated the following three issues: Are there differences in how *fru*<sup>1</sup> males court mature virgin wild-type females compared with how they court mature wild-type males? How does the courtship of a mature virgin wild-type female by male carrying *fru*<sup>1</sup> mutation differ from how a wild-type male does it?

The detected patterns indicate that there was little interaction between the *fru*<sup>1</sup> males and the mature virgin wild-type females and even less between *fru*<sup>1</sup> males and mature wild-type males, but there was more interaction among patterns derived from mature wild-type males and mature virgin wild-type females during courtship.

## 6.2 Experimental procedures

### 6.2.1 Fly stocks

The *D. melanogaster* flies used in this study are of the wild-type Canton S (CS) strain and mutants carrying an allele of the *fruitless* mutation (*fru*<sup>1</sup>) [7,9]. The flies were raised on a cornmeal/agar/molasses/yeast medium at 25°C on a 12:12 L:D cycle. The flies were sexed and the naive males and virgin females were isolated within two hours after eclosion under cold anaesthesia (4°C). The males were kept individually in test tubes until the recording. The mature males and females flies used for the recording were 4-5 days old. Mutant males used in the assays were homozygous for *fru*<sup>1</sup>.

### 6.2.2 Video-recording

A male and a female fly were transferred, by aspiration, into a cylindrical chamber of a mating wheel. Each chamber had dimensions of 0.8 cm diameter and 0.5 cm height. There were four such chambers on each wheel. A 5-min episode (or until copulation in the case of the mature virgins) was recorded for each pair with a Sony Hi8 video/audio camera. The recordings were performed at 25°C and at 75% humidity.

### 6.2.3 Coding of the behaviour

The videotape recordings were first converted from Hi8 video to digital video. The digital video was compressed (~ 90% compression: From 1GB to 60MB) and converted into MPEG 1 files (29.97 frames per second) using Cleaner 6 software (Discreet, New York, NY). The data were analyzed frame by frame (30 frames per second) using an interactive multimedia program. The beginning and ending of every behaviour was systematically registered for the male and female of each pair to a 1/30 of a second resolution. At this time resolution, we discovered novel behaviour elements that were invisible to the unaided eye [Arthur et. al., in prep]. This coded data were then imported into THEME (Noldus, Netherlands) and further processed for pattern detection and analyses. Conditions for the analyses of the courtship data from *fru*<sup>1</sup> males paired with mature virgin wild-type females and with mature wild-type males and also for the mature wild-type virgins paired with mature wild-type males the minimum occurrences were set at 5 and the maximum search level was 3. The significance level was set at  $p = .005$  and Excluded Frequent ET's at 1.5 (i.e. frequency threshold above which patterns involving certain event types are rejected). Mean number of patterns obtained under these conditions are: 106 for real data and 12 for the randomized data for the dyad of mature virgin wild-type females and *fru*<sup>1</sup> males and 68 for real data and 5 for randomized data for the dyad of mature wild-type male and *fru*<sup>1</sup> male; for dyad of mature virgin wild-type female and mature wild-type male the real data gave 531 and the randomized 3 [11]. The behaviour elements coded for are the following:

#### *Female behaviour elements*

*Abdotwist*: a twisting of the abdomen sideways and downwards. *Kicking*: applying tarsi forcefully against partner. *Ovipositor extrusion*: telescoping extension of ovipositor. *Wiggle*: Wagging of abdomen and wings in opposite directions. *Wingflutter*: left and right flicks in succession.

#### *Male behaviour elements*

*Orientation*: Fly is facing the partner, and may involve little motion such as turning to keep facing the partner, leaning over or no motion. *Following*: locomotion in directed pursuit of a partner. *Tapping*: touching partner with tarsi of prothoracic forelegs. *Fencing*: face-to-face and engaged in sparring with prothoracic forelegs. *Wing extension*: wing stretched out away (i.e. perpendicular to the head abdomen axis) from the body biased to the left or right. *Circling*: a sideways skid along a semi-circular path around a partner. *Licking*: proboscis contact with partner. *Abdominal bending (Abdobend)*: abdominal curling under thorax towards the head direction. *Attempted copulation*: abdominal curling directed towards a partner. *Copulation*: sustained genital connection between male and female.

#### *Common behaviour elements*

*Abdominal vibration*: vertical up and down movement of the abdomen. *Decamp*: an evasive jump away from partner, usually involving a somersault. *Grooming*: rubbing of tarsi together, forelegs, midlegs or hindlegs. *Standing*: Absence of locomotion with no

indication of activity directed towards the partner. *Still*: no detectable movement of any body parts during standing. *Walking (walk)*: locomotion that is not directed towards courting the partner. *Wing Flicks*: brief spasmic movement of wings to and from the antero-posterior axis of the body in rapid succession. *Wing Scissoring*: both wings moved away from and back to the body in a rapid scissor-like manner. *Wing Spread*: Both wings are stretched out away from the body to give a wide V-shape forming an angle of at least 90 degrees that is bisected by the anterior to posterior axis of the fly.

## 6.3 Results

### 6.3.1 Novel behaviour elements

We set a criterion that any continuous action that had a beginning and ending that was clearly observable at the temporal resolution of 30 frames per second fit our definition of behaviour element. The new behaviour elements: fencing, abdominal vibration, wiggle, wingflutter, wing flicks, wing scissoring and wing spread were by thus found (See section 6.2.3 for descriptions). The high spatial resolution and sub-second temporal resolution enabled us to observe such fast action behaviour elements.

### 6.3.2 Frequencies of individual behaviour elements

The mature wild-type male displays orientation and following elements during the courting of a mature virgin wild-type female significantly more often than *fru*<sup>1</sup> male courting a mature virgin wild-type female or mature wild-type male. This wild-type male also grooms, walks and stands significantly less often when courting mature virgin wild-type females than *fru*<sup>1</sup> males courting either mature wild-type males or mature virgin wild-type females (Table 6.1.A). There was no fencing observed for mature wild-type males. The only significant difference between how *fru*<sup>1</sup> males courted mature wild-type males and mature virgin wild-type females is that *fru*<sup>1</sup> males displayed more fencing when they courted the mature wild-type males (Table 6.1.A).

Mature virgin wild-type females kicked significantly more often when courted by mature wild-type males than by *fru*<sup>1</sup> males (Table 6.1.B). The experiment consisted of three dyads; a mature virgin wild-type female paired with a mature wild-type male; *fru*<sup>1</sup> male paired with either a mature virgin wild-type female or a mature wild-type male. One-way ANOVA revealed significant differences among the three dyads in 7 out of the 14 behaviour categories: male orientation ( $F_{2,14} = 5.82, p = .017$ ); male following ( $F_{2,14} = 5.9, p = .016$ ); male fencing ( $F_{2,14} = 8.034, p = .006$ ); male grooming ( $F_{2,14} = 14.2, p = .0007$ ); male walk ( $F_{2,14} = 13.75, p = .0008$ ); male standing ( $F_{2,14} = 8.96, p = .004$ ); female kick ( $F_{1,9} = 8.33, p = .02$ ). Subsequent application of unplanned Tukey-Kramer tests uncovered the significance of differences among the members dyads within each category (see Table 6.1 A & B) The differences among the three dyads in the remaining seven categories of behaviour elements were found not to be significant ( $p > .05$ ) [12]. Noteworthy is that the reference to male behaviour in the dyad consisting of the mature wild-type male and *fru*<sup>1</sup> male is intended for the *fru*<sup>1</sup> male.

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|             |           |         |         |      |             |         |          |         |          |
|-------------|-----------|---------|---------|------|-------------|---------|----------|---------|----------|
| orientation | following | tapping | fencing | wing | wing flicks | licking | grooming | walking | standing |
|-------------|-----------|---------|---------|------|-------------|---------|----------|---------|----------|

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|                     | extension   |             |             |             |             |            |            |             |            |             |  |
|---------------------|-------------|-------------|-------------|-------------|-------------|------------|------------|-------------|------------|-------------|--|
| ♂ <sub>f</sub> vs ♂ | 5.6 ± 1.7   | 7.0 ± 0.7   | 14.4 ± 2.9  | 5.4* ± 1.86 | 8.2 ± 3.3   | 4 ± 1.9    | 9.2 ± 2.6  | 10 ± 1.3    | 40.8 ± 7.6 | 28 ± 4.1    |  |
| ♂ <sub>f</sub> vs ♀ | 5.2 ± 2.5   | 6.4 ± 1.3   | 15.4 ± 6.0  | 0.2 ± 0.2   | 16.6 ± 9.9  | 13.8 ± 8.6 | 11 ± 3.9   | 10 ± 1.6    | 58.6 ± 8.4 | 33.2 ± 4.8  |  |
| ♂ vs ♀              | 19.8* ± 5.8 | 24.2* ± 7.0 | 26.8 ± 10.3 | 0.0 ± 0.0   | 31.6 ± 9.54 | 19 ± 8.4   | 12.2 ± 5.8 | 1.6* ± 0.75 | 8.4* ± 3.5 | 8.6* ± 4.13 |  |

♂<sub>f</sub> is *fruitless* mutant male, ♂ is wild-type mature male, ♀ is mature virgin wild-type female. \* -implies statistically significant difference from other entries in row.

**Table 6.1.A** Frequency of display of behaviour elements by *fruitless* mutant male as courter.

|                     | wing flicks | kicking   | decamp     | grooming  |
|---------------------|-------------|-----------|------------|-----------|
| ♀ vs ♂ <sub>f</sub> | 0.2 ± 0.2   | 0.2 ± 0.2 | 2.2 ± 0.37 | 5.4 ± 0.9 |
| ♀ vs ♂              | 8.0* ± 5.4  | 3.2* ± 1  | 2.8 ± 0.9  | 5.4 ± 1.4 |

♂<sub>f</sub> is *fruitless* mutant male, ♂ is wild-type mature male, ♀ is mature virgin wild-type female. \* -implies statistically significant difference from other entries in row.

**Table 6.1.B** Frequency of display of behaviour elements by mature virgin wild-type female as courtee.

### 6.3.3 Which behaviour elements are most involved in patterns ?

To determine the contribution of a behaviour element to the patterns, we expressed the number of patterns in which that particular element occurred as a ratio of the total number of patterns generated for each dyad. We reported the mean of these ratios for every group (See Table 6.2 A & B). One-way ANOVA tests revealed significant differences among the groups for the behaviours: orientation by *fru*<sup>1</sup> male ( $F_{2,14} = 8.2, p = .006$ ); following by *fru*<sup>1</sup> male ( $F_{2,14} = 4.8, p = .03$ ); decamping by *fru*<sup>1</sup> male ( $F_{2,14} = 4, p = .046$ ); fencing by *fru*<sup>1</sup> male ( $F_{2,14} = 6.75, p = .01$ ) [12].

|                     | orientation | following | tapping | fencing | wing extension | wing flicks | licking | grooming | walking | standing | abdobend | decamp |
|---------------------|-------------|-----------|---------|---------|----------------|-------------|---------|----------|---------|----------|----------|--------|
| ♂ <sub>f</sub> vs ♂ | 0.07        | 0.16      | 0.27    | 0.02    | 0.04           | 0.01        | 0.13    | 0.03     | 0.39    | 0.27     | 0.01     | 0.03   |
| ♂ <sub>f</sub> vs ♀ | 0.04        | 0.10      | 0.16    | 0.00    | 0.14           | 0.05        | 0.15    | 0.06     | 0.24    | 0.08     | 0.00     | 0.09*  |
| ♂ vs ♀              | 0.44        | 0.49      | 0.18    | 0.00    | 0.17           | 0.04        | 0.11    | 0.00     | 0.11    | 0.08     | 0.08     | 0.00   |

♂<sub>f</sub> is *fruitless* mutant male, ♂ is wild-type mature male, ♀ is mature virgin wild-type female. \* -implies statistically significant difference from other entries in row.

**Table 6.2.A** Occurrence of individual elements as fractions of total number of patterns detected from *fruitless* mutant male courter (mean of individual ratios).

|                     | wing flicks | kicking | decamp | grooming | Standing | walking |
|---------------------|-------------|---------|--------|----------|----------|---------|
| ♂ vs ♂ <sub>f</sub> | 0.01        | 0.00    | 0.00   | 0.03     | 0.10     | 0.20    |
| ♀ vs ♂ <sub>f</sub> | 0.00        | 0.00    | 0.00   | 0.01     | 0.40     | 0.42    |
| ♀ vs ♂              | 0.01        | 0.03    | 0.00   | 0.01     | 0.15     | 0.42    |

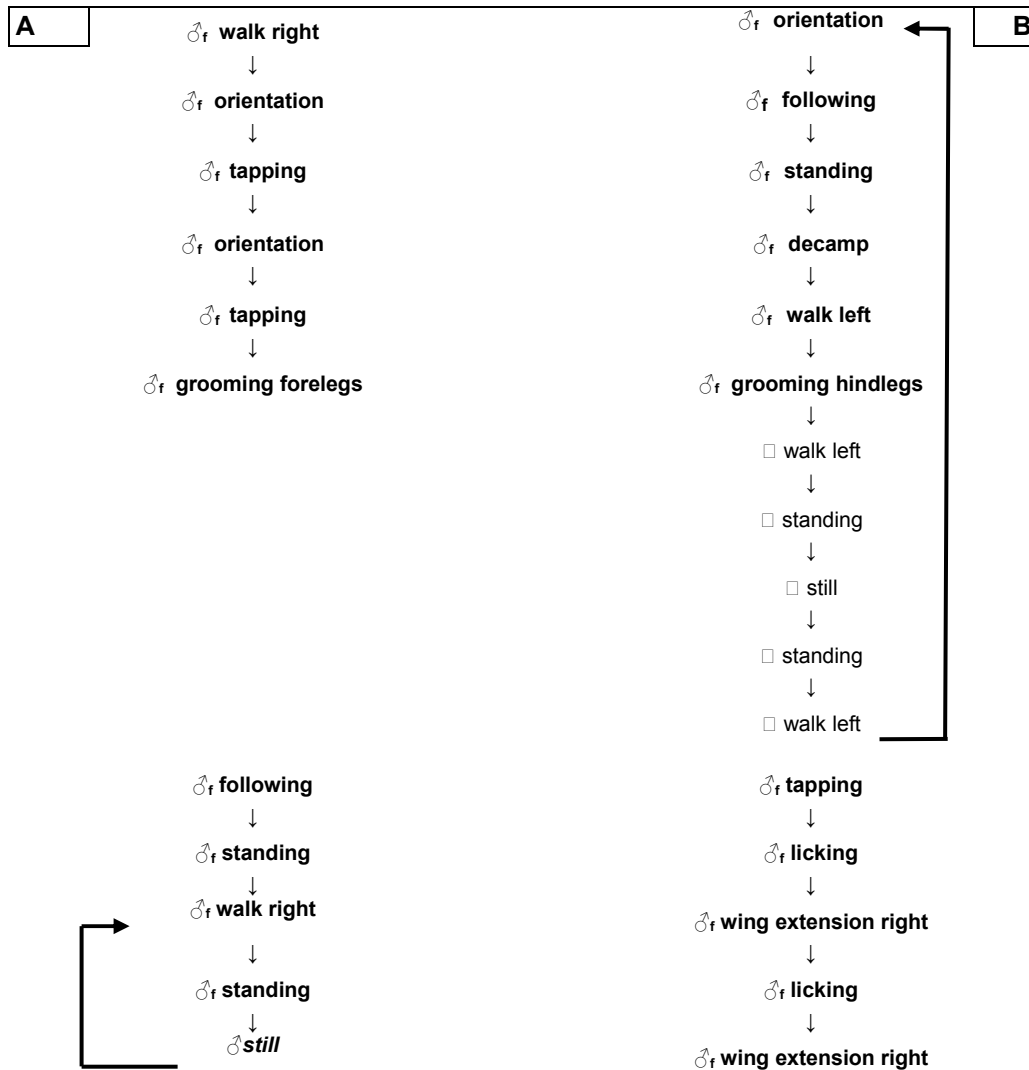
♂<sub>f</sub> is *fruitless* mutant male, ♂ is wild-type mature male, ♀ is mature virgin wild-type female.

**Table 6.2.B** Occurrence of individual elements as fractions of total number of patterns detected from mature virgin wild-type female (mean of individual ratios).

### 6.3.4 Characteristic interactions within patterns of each dyad group

Patterns obtained within each dyad group were aligned to each other to construct a pattern that is common within each dyad group. This gave an idea of how the elements of the courter and courtee interact with each other. The *fru*<sup>1</sup> male and mature wild-type male dyad

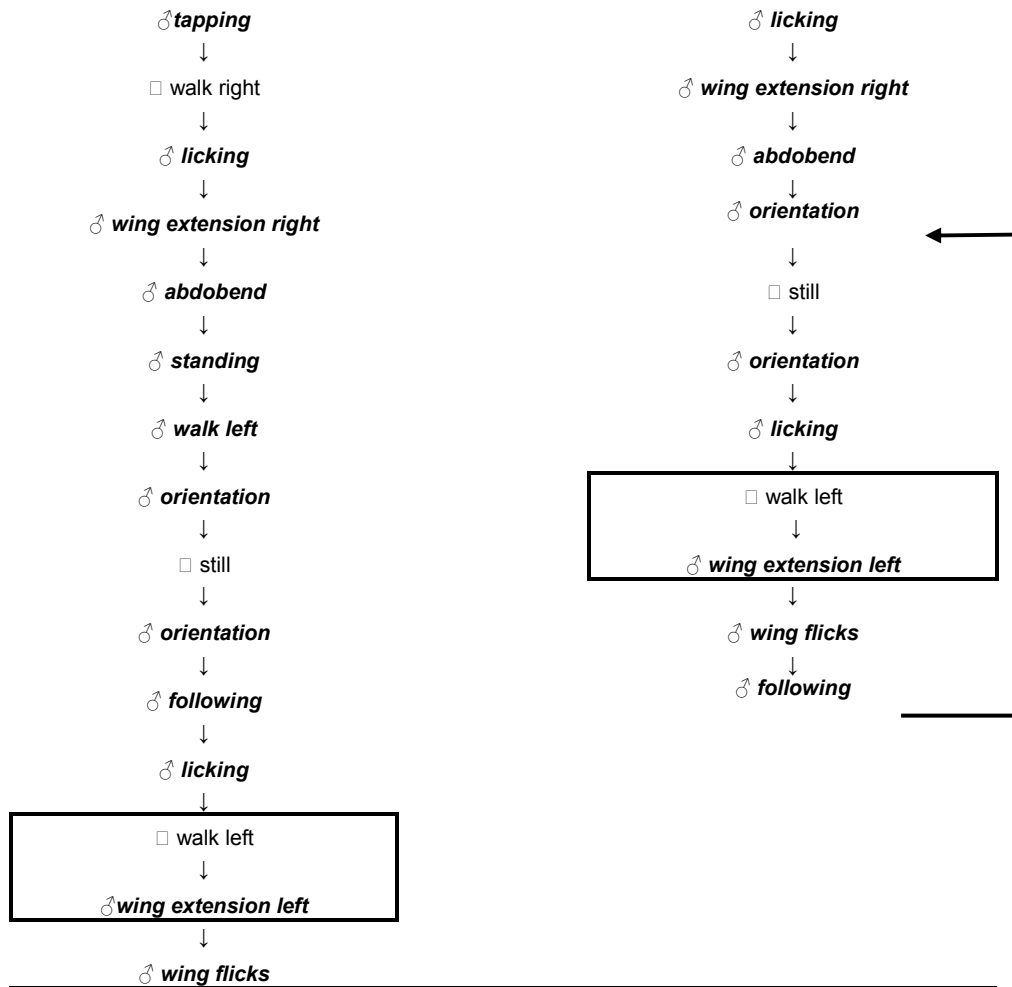
group shows for the common pattern that: i) elements of *fru*<sup>1</sup> male are not interspersed with those of the mature wild-type male. ii) there is only one element contributed by mature male (Fig. 6.1A). The *fru*<sup>1</sup> male and mature virgin wild-type female dyad group shows for the common pattern that: i) elements from *fru*<sup>1</sup> male and mature virgin wild-type female do not intermingle ii) mature virgin wild-type female contributes about a third of the elements of the pattern (Fig. 6.1B). Finally, the mature wild-type male and mature virgin wild-type female dyad groups show that for the common pattern: i) the elements of the female are widely distributed among the male's; ii) female contributes about a third of the elements of the pattern; iii) there are characteristic pairings of elements, with one from the male and the other from the female, in each case (Fig. 6.2).



♂<sub>f</sub> is *fruitless* mutant male with elements in bold print, ♂ is wild-type mature male with elements in bold italics, □ is mature virgin wild-type female with elements in normal print.

**Figure 6.1** Common patterns derived from aligning and selecting the overlapping pattern that occur in each dyad within a pairing group with *fruitless* mutant male as a courter A. Pairing of *fruitless* mutant male and a mature wild-type male B. Pairing of *fru*<sup>1</sup> mutant male with mature virgin wild-type female.





$\text{♂}_f$  is *fruitless* mutant male with elements in bold print,  $\text{♂}$  is wild-type mature male with elements in bold italics,  $\square$  is mature virgin wild-type female with elements in normal print. Two elements, one from each partner that appear always appear together are enclosed in the rectangles.

**Figure 6.2** Common patterns derived from aligning and selecting the overlapping pattern that occur in each dyad within a pairing group of mature virgin wild-type female and mature wild-type male.

## 6.4 Discussion

Our approach enabled us to discover novel micro-behaviours that are invisible to the unaided eye at normal temporal resolution. We showed that high frequency of a behaviour element is no guarantee that it would contribute to a pattern. Furthermore, the analysis using THEME revealed some structures of the courtship behaviour architecture that were not apparent to the observer: It appears that *fru<sup>1</sup>* males did not interact with their partners in sustained activity that was long enough to generate an integrated pattern (i.e. there was total segregation of the elements contributed by each partner to the common pattern for the dyad group). A similar result was obtained using nonlinear methods to extract salient components from the same data set [13]. This suggests that courtship display of *fru<sup>1</sup>* male depends less on cues from the partner. Thus *fru<sup>1</sup>* males require only a short trigger to initiate the male courtship program. The *fru<sup>1</sup>* male displayed courtship behaviour similarly towards wild-type female or male partners, except that the females contributed more elements to the common pattern. Wild-type females contribute equally (i.e. about a third of elements) when courted by *fru<sup>1</sup>* males or by wild-type males, but there is more interaction and wider distribution between the elements from wild-type males and females in a pattern

(i.e. better integration). Characteristic pairings of some elements displayed by the dyad of wild-type male and female are suggestive of a type of syntax used in the communication.

Our findings show that this approach provides a new and deeper insight into the interaction and contribution of each partner in a dyad. The next steps would be to develop quantitative methods to compare patterns on basis of length and diversity of elements that compose the patterns, to develop a measure of complexity of the patterns and to decipher the grammar of a putative syntax. This would provide an even more detailed account of the courtship behaviour and a basis for comparison between different dyad groups.

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