Circular dispersal of larvae in the necrophagous Diptera
Protophormia terraenovae (Diptera: Calliphoridae)

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Abstract. Behaviour of the larval dispersal of Calliphoridae flies prior pupation was often noted in the literature but rarely investigated statistically. The aim of this study was to explore with original mathematical tools such larval dispersal of a necrophagous Diptera: Protophormia terraenovae (Robineau-Desvoidy 1830) (Calliphoridae, Chrysomyiinae). The study, performed in South-West France during summer, gives preliminary results in outdoor experimental conditions. The shape of the dispersal is circular and has a concentric distribution around the feeding zone. Puparia were most often found in a radius of 15 cm and circular statistical analysis showed that no preferential direction was taken by the larvae. The maximum distribution density was related to the feeding zone and the location of the oviposition site can be extrapolated.

Résumé. Dispersion circulaire des larves chez le diptère nécrophage Protophormia terraenovae (Diptera : Calliphoridae). Le comportement de la dispersion larvaire des mouches Calliphoridae avant la pupaison est souvent noté dans la littérature, mais il est rarement étudié statistiquement. L’objectif de ce travail est d’explorer par une approche mathématique originale la dispersion du Diptère nécrophage Protophormia terraenovae (Robineau-Desvoidy 1830) Calliphoridae, Chrysomyiinae. Cette étude a été menée in natura dans le Sud-Ouest de la France durant l’été. La forme de l’aire de dispersion est circulaire et concentrique autour de la zone de nourrissage des larves. Les pupes se trouvent le plus souvent dans un rayon de 15 cm ; les analyses de statistiques circulaires menées montrent qu’il n’y a pas de direction privilégié par la larve durant sa phase de déplacement. La densité des pupes correspondant au maximum de distribution est en relation avec la source de nourriture des larves mobiles et il est ainsi possible de déterminer le lieu initial de la ponte.

Keywords: Forensic entomology, circular statistics, migration, distribution, larval dispersal.

Necrophagous Diptera are the first and the most important insects of entomological successions on a cadaver. Females lay eggs on wounds or natural orifices (Haskell et al. 1997). The larvae are meat consumers, giving them a close relation with corpses. Nevertheless, we know that most necrophagous Diptera larvae have a wandering stage (or post-feeding stage) (Campobasso et al. 2001). This occurs between the larval growth stages and the pupal stage. During this stage, the larvae seem to be escaping from the growth medium where bacteria and fungi proliferate and searching for a site to pupate (Haskell et al. 1997).

Greenberg (1990) had shown that in indoor conditions, Chrysomyiinae seem to have a short dispersal distance. Furthermore, several field observations describe Protophormia terraenovae pupating on or close to cadavers (Cragg 1955; Norris 1959; Greenberg 1991; Benecke 1998). Other studies describing the dispersal of blowflies were made, however data were always recorded from emerging flies on a large scale or on some indoor linear design (Travis et al. 1940; Godoy et al. 1995; Godoy et al. 1996; Tessmer & Meek 1996).

Puparium positions indicate the exact location where larvae stop their dispersal. Moreover, chitin from dry exuviae is resistant to chemical attack and microorganisms (Reiter & Wollenek 1985). Long after death, puparia can persist on or close to the remains of the cadaver (Reiter & Wollenek 1985). Some have been found in graves 2700 years old and in prehistoric houses (Nuorteva 1987) or in hollow bones from the Tertiary era (Teskey & Turnbull 1979). So, they are usable long after the emergence of flies and information is preserved in the soil. This is why we chose to work on puparia from P. terraenovae remaining in soil after the emergence of adults.

The first aim of this study was to determine whether the dispersal type of P. terraenovae in outdoor experimental conditions corresponded to that described for Chrysomyiinae by Greenberg (1990), i.e., a great
proportion of larvae with a short dispersal distance.

The second aim was to evaluate if pupal distribution in soil may be used as a tool to locate the starting point of a circular dispersal, i.e., a source of meat or a wound.

In order to evaluate these parameters, an original approach using circular statistics was performed and some results are compared with former studies done on closely related species.

Material and methods

Diptera

The species used for the study is *Protaphormia terraenovae* (Calliphoridae, Chrysomyiinae). It was chosen for its ubiquitous geographical distribution. Furthermore, *P. terraenovae* possesses a “sedentary life style” as puparia of this species have often been described on cadavers or very close to them (Cragg 1955; Norris 1959; Greenberg 1991; Benecke 1998).

The specimens used in the experiment were obtained from a population from Villeneuve-de-Rivière (south-west of France). The blow flies were reared at ambient temperature (18-24°C).

Searches were performed only to 10 cm depth because, larvae of *P. terraenovae* deposit puparia under the soil (Cragg 1955). A Cartesian point was installed. The origin was chosen as the centre of the piece of meat and an orientation was given according to the points of the compass. The blow flies were reared at ambient temperature (18-24°C).

The blow flies were reared at ambient temperature (18-24°C). Sugar, water and fresh minced beef were available *ad libitum* for adults. Larvae were reared on minced beef lying on wet peat. Site

The field study was conducted in a flat leafy wood of 3 to 4 hectares on University Paul Sabatier campus in Toulouse (South-west France). It was a former swampy area drained for the building of the university in the 1960’s. The soil is mainly composed of hard core and is relatively dry. Litter is not very abundant (1 to 2 cm thick) and heterogeneous.

Experimentation

The experiment was performed in three steps: 1. Field preparation, 2. Larva deposit and time for dispersal and 3. Excavation of soil in search for puparia.

Before each replicate, surface litter was removed from a zone of three square meters. Young growth of trees, ivy and brambles were uprooted so that their root network would not make it too difficult for later excavation and for maximum standardisation.

Sets of 200 to 350 larvae (L2) were laid on soil and covered by 100 grams of fresh minced meat (12 x 8 cm area) to allow them to complete their growth. To restore an undergrowth habitat and not disturb the lucifugous larvae (Bass 1997), the areas were covered by a thin coat of litter to protect larvae during migration and to avoid them burrowing too deep into the soil (Cragg 1955). A Cartesian point was installed. The origin was chosen as the centre of the piece of meat and an orientation was given according to the points of the compass. The experimental area was covered by a cage draped over with a mosquito net pushed slightly into the soil to prevent disturbance by vertebrate scavengers (Greenberg 1991; Godoy et al. 1996; Bass 1997; Galloway 1997; Campobasso et al. 2001) and to limit the impact of other dipterans and parasitism.

After migration had occurred, puparia were excavated. Searches were performed only to 10 cm depth because, larvae of some blowfly species never dig under 5 cm (Travis et al. 1940). The Cartesian coordinates of each puparium were noted.

Between March and August 2002, 10 replicates of a single site (i.e., only one point of release) were carried out. To test the potential interaction between several sites (attraction or repulsion), 4 replicates with a double site (i.e., two points of release) were performed. A first type where the centres of the two pieces of meat are placed 26 cm apart (i.e., 14 cm free space between edges) and a second type with centres at 15 cm from each other (3 cm between edges).

Statistical analysis

Cartesian coordinates were recorded under Excel. Migration direction was evaluated by calculation of a mean vector defined by a mean angle ($\alpha$) and a mean length ($r$) (Zar 1999) calculated with the position of each point.

$$\cos \alpha = X / r \text{ or } \sin \alpha = Y / r$$

with

$$X = \sum \cos \beta_i / n \text{ and } Y = \sum \sin \beta_i / n$$

$$r = \sqrt{(X^2 + Y^2)}$$

Where $n$ is the number of individuals and $\beta$ the dispersal angle of each individual ($i, i+1, ..., i$).

The significance of the length of the mean vector was tested with the Rayleigh test ($z$) (Zar 1999).

$$z = R^2 / n \text{ or } z = nr^2$$

where $R = nr$

Distribution of puparia (random, aggregate or regular) was tested with two methods. The first was the Clark & Evans test (R) (Clark & Evans 1954) with its standard variant of the normal curve (c) (Mather 1947). It is especially suitable to test population distribution, but it forces us to choose an arbitrary work area.

$$R = r_s / r_e \text{ with } r_s = \Sigma r / N \text{ and } r_e = 1 / 2 \sqrt{e}$$

$$c = (r_s - r_e) / \sigma_r$$

Where: $r_s$ is the mean distance of the series of distances to nearest neighbour, $r_e$ the mean distance to nearest neighbour expected in a large random distribution of density rho.

$$r = \text{distance between points } 2 \text{ by } 2.$$  

$$N = \text{number of individuals}.$$  

$$e = \text{population density} \text{ (here based on a circle } 120 \text{ cm in diameter}).$$  

$$\sigma_r = 0.26136 / \sqrt{Ne} \text{ is the standard error of the mean distance to nearest neighbour in a randomly distributed population of density rho.}$$

In a random distribution, $R = 1$. Under maximum aggregation, $R = 0$ (all individuals occupy the same locus). $R$ reaches the maximum value of 2.1419 when field occupation is regular and optimum. Circular distribution analysis and the Clark and Evans test were performed under Excel.

The second method was used to calculate deviation from normality for distribution frequencies characterised by two indices; Skewness (G1) and Kurtosis (G2) (SYSTAT 8.0 1998) (Sokal & Rohlf 1995).

A first calculation gives pair distances between the different locations of pupation, and a second, the distance from each of these locations to the central point of our experimental design.

To compare replicates, data were normalised by decimal logarithm transformation ($\log (d+1)$) and tested with the Kolmogorov-Smirnov nonparametric test (Lilliefors method) (SYSTAT 8.0 1998). An ANOVA was performed and the post-hoc Bonferroni test used (SYSTAT 8.0 1998). 3D-illustrations were obtained with Surfer software (SURFER 2002). The Krigeage option was used as interpolating method.
**Results**

Out of 14 replicates, only 5 with single sites and 2 with a double site were used for the study, the others were discarded due to the destruction of site by scavengers or due to the few puparia found (mortality being due to brusque variations of temperature). The remaining 7 replicates are named as follows in Table 1. During excavation, puparia were rarely found beyond 30 cm and never more than 100 cm from the origin.

Table 1. Nomenclature of replicates with code used in the text, duration of experiment and the initial number of laying sites installed. The double site replicate of “July 2” was used as two single laying sites (no interaction found). East and West denomination were applied depending on their position with respect to the Cartesian point.

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Duration</th>
<th>Number of laying sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>May 1</td>
<td>2002-05-01 to 2002-05-15</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>May 2</td>
<td>2002-05-16 to 2002-05-31</td>
<td>1</td>
</tr>
<tr>
<td>Jn1</td>
<td>June 1</td>
<td>2002-06-01 to 2002-06-15</td>
<td>1</td>
</tr>
<tr>
<td>Jn2</td>
<td>June 2</td>
<td>2002-06-16 to 2002-06-30</td>
<td>1</td>
</tr>
<tr>
<td>Jy1</td>
<td>July 1</td>
<td>2002-07-01 to 2002-07-15</td>
<td>1</td>
</tr>
<tr>
<td>Jy2</td>
<td>July 2</td>
<td>2002-07-16 to 2002-07-31</td>
<td>2</td>
</tr>
<tr>
<td>Jy2e</td>
<td>July 2 east</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Jy2w</td>
<td>July 2 west</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>August</td>
<td>2002-08-01 to 2002-08-15</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2 shows the number of puparia found for each replicate. A great majority of puparia were found under the area covered by the meat (fig. 1).

The two sites of “July 2” did not show any interactions (fig 1.b) and can be used as two independent replicates. For “August”, interactions were observed between the two sites. Actually, more than 350 puparia were found next to the east site though only 250 larvae had been placed there. So, more than 100 larvae from the west site had migrated to the east site (fig 1.c).

**Migration**

The results of the migration direction test are given in table 3. Z-values of the Rayleigh test give an evaluation of whether circular dispersion from a central point is homogeneous or not. When not, the resulting dispersion vector can be oriented indicating a preferential direction. In all replicates except one (M2) larval populations took a “preferential” direction and a vector was determined.

Only the population of the M2 replicate had a statistically uniform distribution but in reality, there was over-dispersal in the southern half (60 cm vs. 30 cm in the north part, data not shown). Figure 1 shows that despite this significant preferential direction, the dispersal occurs quasi-radially.

Tessmer & Meek (1996) have already investigated the field of dispersal of some Diptera in different media. Comparison (tab. 3) with their results obtained in woodland gives a fair agreement. However, we did not find the seasonal preferred directions described by these authors but the duration of our experiment was shorter and with a different species.
The results of the two different statistical methods used for the evaluation of distribution of puparia over a radius of 60 cm are given in table 4. These tests determine whether individuals have a random, aggregated or regular distribution. All of them gave the same results but with subtle differences in some cases.

The Clark & Evans test, based on the nearest neighbour, indicates that not all replicates were randomly distributed. All had an aggregated distribution except for replicate M2. For this replicate, the test indicates that the distribution was similar to a regular distribution.

Dissymmetric analysis of normal curves (Skewness: G1) showed that replicates did not show a normal distribution. For all replicates G1 > 0, so short distances were predominant when comparing puparium locations 2 by 2, but also when checking between the puparia and the origin. Therefore, individuals had an aggregated distribution. These aggregations were more pronounced from July (tab. 4).

Curves generally had a leptokurtic pattern (G2 > 0) (Sokal & Rohlíř 1995). This indicates that one interval of values was overrepresented. These intervals were always below 15 cm for distances from the origin (intervals most represented: 0 to 5 cm, 5 to 10 cm or 10 to 15 cm) (data not shown). Therefore, puparia were aggregated around the origin and concentrated in the first 15 centimetres.

Comparison of replicates

After normalisation of the data (see Material and Methods), populations were compared with an ANOVA test. Data sets of distances of puparia were tested 2 by 2 and then we tested the data set of distances of puparia from the origin.

First, a 2 by 2 test on distances between puparium locations gives:

\[
F_{6; 111540} = 13309 \quad (P < 10^{-5}); \quad R^2 = 41.7\%
\]

With: 6 = (number of replicates – 1) and 111540 = number of distance pairs used in the test. This test shows that variance was explained for 41.7% (R²) by differences of pairs of distances between puparia. The rest of the variance can be due to micro conditions (soil type, obstacles, moisture, temperature, etc.). The Bonferroni test shows that all replicates were different. Therefore, distributions were not exactly reproducible from one replicate to another.

For distances of puparia to the origin, the results are the following:

\[
F_{6; 1137} = 65.53 \quad (P < 10^{-5}); \quad R^2 = 25.7\%
\]

The Bonferroni test indicates that replicates Jn1, Jn2, Jy1, Jy2e and Jy2w are not significantly different. So, larvae covered the same distances during migration for these replicates.

Discussion

As expected, *Protophormia terraenovae* presented a dispersal of “type 3” as described by Greenberg (1990), *i.e.*, nil or short migration distances. Directions were not strongly enough marked to obtain a patch of puparia far from the origin. So, the origin is integrated in the scatter of points with a radial distribution of
puparia around it. Even though migration directions have been demonstrated, they changed a lot from one replicate to another without any preferred direction. Micro variations (soil density, exposure to sunlight, hygrometry, etc.) could be responsible. As possible sources of heterogeneity, some dry or compact zones were observed where no puparia were found. This kind of situation was observed for population M2 where only few puparia were found in the north east quarter, in a zone where the soil was particularly compact (data not shown). Moreover, Greenberg (1990) noted that larvae can detect the soil density and migrated farther than ordinary on hard ground.

Puparia presented an essentially aggregated pattern with a decreasing density gradient from origin to edge. No variations in the frequency of pupae as a function of distance from the origin was observed as for Cochliomya macellaria (Calliporidae) (Von Zuben et al. 1996; Boldrini et al. 1997). Several potential ecological adaptations are perhaps at the origin of this kind of distribution. In some species, larvae were able to detect larval density and induce a farther migration (Boldrini et al. 1997; Gomes & Zuben 2005). However, we noted a trend for puparia to be aggregated when the level of recovery was high and a more regular distribution as soon as puparia recovery was poorer without longer migration. So, in this situation, the larval density was not involved. Larvae seem to have constant short range dispersion under or close to food source. It may have an adaptative explanation regarding the exposition to predators or parasitoids (Gomes & Zuben 2005). Two cases arise, the first one occur in long rang migration during which larvae are expose to its natural enemies (Greenberg 1990) but go faraway of food remains that are attractive for predators and parasitoids (Godoy et al. 1995). In the second case, larvae stay under or close to remains. The proliferation of other larva from following necrophagous waves can also hide their odour or reduce the probability for one individual to be found in the mass of potential prey or host. It may depend on biological characteristics and methods of patch exploitation of their specialist or generalist predators and parasitoids. The proximity of remains cans also providing higher temperature for a faster maturation of pupae and/or preserving them of low autumal and springlink negative temperatures.

The second hypothesis was that pupal distribution could be used to locate the global start area of migration. It is clear that using our 3D design we may build up a topological map of puparia aggregation. It gives us a point of maximum density that is always included in the area covered by meat. In conditions of no exhaustion of feeding source, this area is comparable to a growing site from which prepupal larvae start migration.

Our double site replicate showing interactions (“August”) is indicative of the minimal distance between two laying sites to apply our analysis. The overabundance of food in this experimental design, exclude the hypothesis of migrations in search for additional food. Further study will be necessary to identify the exact minimal distance between two laying sites below which the two scattered sets of points can be distinguished.

### Table 4. Distribution of recovered puparia: Clark & Evans test and Skewness and Kurtosis analysis.
Clark & Evans test (R values and standard variate c) with his interpretation: A (aggregated) or U (uniform) with 99% confidence level. Skewness and Kurtosis analysis (± se) distortion of normal distribution for two series of data: distances between puparia taken 2 by 2 and distances of each puparium from the origin. Grey boxes: not significant. Values were not calculated for the August double laying site replicate.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>Jn1</th>
<th>Jn2</th>
<th>Jy1</th>
<th>Jy2e</th>
<th>Jy2w</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clark &amp; Evans test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>index R</td>
<td>0.87</td>
<td>1.18</td>
<td>0.73</td>
<td>0.87</td>
<td>0.10</td>
<td>0.41</td>
<td>0.43</td>
<td>0.17</td>
</tr>
<tr>
<td>Standard variate (c)</td>
<td>–2.64</td>
<td>2.67</td>
<td>–6.25</td>
<td>–4.17</td>
<td>–26.52</td>
<td>–12.24</td>
<td>–15.15</td>
<td>–32.1</td>
</tr>
<tr>
<td>A U A A A A A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Skewness (G1)</strong></td>
<td>0.964</td>
<td>0.710</td>
<td>0.946</td>
<td>0.434</td>
<td>2.563</td>
<td>1.782</td>
<td>1.449</td>
<td></td>
</tr>
<tr>
<td>± se</td>
<td>0.031</td>
<td>0.059</td>
<td>0.023</td>
<td>0.013</td>
<td>0.014</td>
<td>0.018</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td><strong>Kurtosis (G2)</strong></td>
<td>–0.144</td>
<td>–0.020</td>
<td>–0.239</td>
<td>11.770</td>
<td>4.030</td>
<td>2.233</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± se</td>
<td>0.061</td>
<td>0.118</td>
<td>0.047</td>
<td>0.025</td>
<td>0.029</td>
<td>0.035</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td><strong>Skewness (G1)</strong></td>
<td>1.319</td>
<td>1.313</td>
<td>1.686</td>
<td>0.114</td>
<td>2.420</td>
<td>2.044</td>
<td>2.075</td>
<td></td>
</tr>
<tr>
<td>± se</td>
<td>0.226</td>
<td>0.311</td>
<td>0.199</td>
<td>0.147</td>
<td>0.156</td>
<td>0.177</td>
<td>0.223</td>
<td></td>
</tr>
<tr>
<td><strong>Kurtosis (G2)</strong></td>
<td>2.155</td>
<td>0.883</td>
<td>1.970</td>
<td>–0.396</td>
<td>12.890</td>
<td>4.344</td>
<td>5.567</td>
<td></td>
</tr>
<tr>
<td>± se</td>
<td>0.449</td>
<td>0.613</td>
<td>0.395</td>
<td>0.294</td>
<td>0.312</td>
<td>0.352</td>
<td>0.442</td>
<td></td>
</tr>
</tbody>
</table>
Results must be considered with caution as we showed that some uncertainties remain as to the exact point of origin. We also showed that when puparium density is low, distribution may vary. We are convinced that our experimental design could present biases, but we did find that the small distances covered by larvae allowed us to identify a growing area.

Among various possible sources of bias, factors such as predation, competition, and soil heterogeneity should be taken into account. The heterogeneity of a natural field can cause many artefacts or cause distribution patterns other than those observed during our study. Fallen branches on soil can cut the migratory path of post-feeding stage larvae. Puparia could then be found distributed with a linear pattern along the obstacle.

Even so, these results are interesting in forensics. Indeed, they may reveal the laying site of these flies. More often, laying sites correspond to natural orifices (around the head and pelvis) or wounds because neither adults nor larvae are able to puncture healthy skin (Haskell et al. 1997). Therefore, in cases where the cadaver is highly decomposed or totally reduced to a skeleton, the position of puparia in the soil provides information that might indicate the presence and location of any injuries. In real cases, things are more difficult, since many factors can disturb the behaviour of larvae during feeding and post-feeding stages, but it will be interesting to pursue this line of investigation.

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