

# Analysing aphid behaviour with time-to-event techniques to discriminate between susceptible and resistant plants

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Many studies address aphid feeding behaviour to learn more about resistance mechanisms in plants. Time-to-event techniques can be used to exploit the datasets more profoundly by looking at changes in aphid behaviour over time. Our present dataset was acquired by automated video-tracking of probing behaviour of *Nasonovia ribisnigri*, the lettuce aphid, on leaf discs of two different lettuce cultivars, one susceptible to aphids and one resistant to aphids. The behaviour of individual aphids was recorded in a no-choice situation during 8 h. All experiments were performed on three different days. We first addressed the structure of the alternating non-probing and probing behaviours. To investigate whether the duration of these two behavioural components is affected by plant resistance, we performed a time-to-event analysis for the events 'start of a probe' and 'end of a probe'. These analyses showed that the tendency to stop a probe was lower for aphids on the susceptible cultivar and the tendency to start a probe was higher on that cultivar. This can be seen in the durations of the probes – these were longer on the susceptible cultivar – and the non-probes – these were shorter on the susceptible cultivar. For the current dataset we analyse the behaviour in more detail by including some covariates. In the future, we will use the time-to-event analysis to assess the level of resistance of varieties of a plant species by considering the distributions of both probe and non-probe durations.

*Keywords:* survival analysis, aphid behaviour, host plant resistance

Aphids are major pests for crops all over the world. They limit plant productivity by feeding on the phloem. Moreover, aphids are often vectors of plant pathogens. Almost every crop is a host to at least one aphid species. Because

most aphid species developed resistance to insecticides, they are difficult to control (Blackman & Eastop 2006, Minks & Harrewijn 1989, Powell *et al.* 2006). In addition, the lifestyle of aphids can hamper the efficiency of chemical control, because they may feed only on the occluded inner leaves of a plant (Palumbo 1999). Plants have evolved multiple ways to defend themselves against herbivorous insects, for instance, by means of repellent volatiles, trichomes, toxins and digestibility reducers (Panda & Khush 1995, Schoonhoven *et al.* 2005, van Poecke 2007, Dicke & Baldwin 2010). Exploiting natural variation in host plant resistance to control herbivorous insects is one of the basal elements of environmentally benign pest management (Panda & Khush 1995, Schoonhoven *et al.* 2005).

Previously, it has been demonstrated that plant resistance can be inferred from the behaviour of aphids (Pickett *et al.* 1992, Klingler 1998, Pompon & Pelletier 2012). Aphids are phloem-feeding insects. When probing a plant with their stylets aphids can ingest primary and secondary plant metabolites. Before aphids start feeding, they make multiple short exploratory probes, without extracting phloem from the plant. Stylet penetrations take at least 10 min before a phloem vessel is reached (Tjallingii in Minks & Harrewijn 1988) and often the first event of phloem uptake occurs several hours after initial contact between aphid and plant. From previous research it is known that resistant plants induce more short probes compared to susceptible plants. Also the total time spent on phloem ingestion is much shorter on resistant compared to susceptible plants (Cook *et al.* 1987, Montllor & Tjallingii 1989, Sauge *et al.* 1998).

To record the behaviour of aphids, we used an automated video-tracking method. This method is less precise than Electrical Penetration Graphs (EPG), but comparable to human observations of probes. With video-tracking the position and movements of the insect were recorded with a sample rate of 25 frames per second during the whole recording period. By registration of moving and non-moving events and the position of the aphid (on the leaf or elsewhere in the arena), the video tracking software EthoVision® XT 8.5 acquired data about the number, duration and timing of probes (Noldus *et al.* 2002, Kloth *et al.* in prep.). Unlike EPG this method cannot verify physical contact between stylet and plant, which plant tissue is penetrated and whether any phloem is ingested. The advantage of automated video-tracking is, however, that it can screen many more plant lines in parallel in a shorter time compared to EPG. A previous study showed that number and durations of probes measured with this video-tracking system were highly correlated to human observations of probes (Kloth *et al.* in prep).

In this study the behaviour of aphids is analysed by survival analysis. Survival analysis has not been used a lot to analyse behavioural data, but is suitable for this kind of data (Velema *et al.* 2005). Usually the total time spent on a certain behaviour is recorded, but not if this is one consecutive period or multiple shorter periods. Survival analysis works with time intervals in which this

difference can be visualized. Another advantage of survival analysis is the fact that censored data, i.e. data where the behaviour is still going on at the end of the observation, is also included in the analysis (Jansen *et al.* 2004). In addition, both probing and non-probing events can be assessed with survival analysis, while non-probes have been ignored in most studies.

The aim of this study is to investigate whether survival analysis is a suitable method for identifying resistant and susceptible plants in a dataset acquired by automated video-tracking. To this end, we use video-tracking observations of the lettuce aphid, *Nasonovia ribisnigri* biotype Nr:0, on a resistant and susceptible cultivar of lettuce, *Lactuca sativa*.

## METHODS AND MATERIALS

### Data

We observed winged lettuce aphids and their feeding behaviour on either a lettuce cultivar known to be very resistant (Corbana) to this aphid species or a lettuce cultivar known to be susceptible (Terlana) to this aphid species (ten Broeke *et al.* 2013). The probing behaviour of the aphid was recorded using automated video-tracking. Each aphid was observed in a circular arena, 8 mm in diameter, containing a leaf disc, 6 mm in diameter, with the abaxial side up on a substrate of agar. The duration of each experiment was 8 hours during which the position and probing behaviour of the aphid was continuously recorded with a camera and EthoVision® XT 8.5 (Noldus *et al.* 2002, Kloth *et al.* in prep.). For both the susceptible and resistant lettuce cultivars 30 replicates were performed, each with a different aphid and plant individual. Both cultivars were screened simultaneously over 3 days.

### Time-to-event analysis

In general, time-to-event analyses (or survival analyses) have the following setup. First, the event of interest is defined. Thereafter, the time until the occurrence of this event is recorded. The time until an event happens is called the failure time. Sometimes, these failure times cannot be observed because an individual is out of sight and it is unknown what has happened. Such observations are censored observations. Censoring can also occur due to the fact that an experiment stopped before the event of interest has happened. Based upon the failure times, we analysed the probability per unit time that the event will happen given that it has not occurred yet. This conditional probability is called the hazard rate  $[h(t,z)]$  and can vary in time  $t$  with different influencing factors or covariates  $z$ . Originally, the considered event is death, hence the term ‘survival analysis’, and this event can only happen once to each individual.

In the current dataset, we are interested in probing behaviour of aphids. With the observed and censored probing and non-probing durations we made the histograms for probing and non-probing on susceptible and resistant plants. We

also calculated a crude way for determining the mean duration of (non-)probes, namely the total time of all (non-)probe lengths divided by the total number of realised (non-)probes. For the different durations we also made Kaplan-Meier survivor curves (Klein & Moeschberger 2003) on susceptible and resistant plant species to show the effect of cultivar on the durations.

To assess the qualitative effect of the covariate ‘plant cultivar’ (coded as 0 for Terlana and 1 for Corbana) we used Cox proportional hazards model, where the effect  $\beta$  of covariate  $z$  is expressed in the factor with which the baseline hazard is multiplied:  $h(t,z) = h_0(t) \exp(\beta z)$ .

In this study, we consider two events of interest: the beginning of a probe and the end of a probe (analogous to how behavioural records are analysed by Velema *et al.* 2005). In contrast to conventional survival analyses, the events of interests can happen for the same individual multiple times during a period of 8 h of automated video-tracking. The two events, i.e. the start and end of a probe, are analysed in two separate Cox proportional hazards models by respectively analysing the non-probe and probe duration. The only covariate considered in the current paper is whether the aphid feeds on a susceptible (cultivar Terlana) or resistant (cultivar Corbana) host plant. For the analysis of probe duration we used the tendency to stop probing on a susceptible plant as the baseline hazard rate. In both the probe duration and the non-probe duration data, right censoring occurs because the observations are stopped at a fixed time point.

## RESULTS

The only different behavioural bouts that we addressed in the video-tracked behaviour of the aphids are the probing and non-probing bouts. Probing bouts are the intervals between the start and the end of the probing behaviour, while non-probing bouts are the complementary intervals, namely between the end of one probe and the start of the next. Because EthoVision® XT 8.5 is programmed to register a probe start on the condition that the aphid was not moving over a consecutive period of several seconds before the start, each behavioural record starts by definition with a non-probing bout.

We have analysed the non-probing bouts first by making a histogram of all censored and observed probing bout lengths on the resistant and susceptible cultivars (Figure 1a,b). We performed a similar analysis on the non-probing bouts by making a histogram of all censored and observed non-probing bout lengths on the resistant and susceptible cultivars (Figure 1c,d). Because there are so many non-probes and probes with a short duration these histograms do not clearly show the differences between the cultivars. Therefore, we provide in Figure 2 the log of the Kaplan-Meier survivor curves. In panel (a) of Figure 2 it becomes clear that the probing bouts in the susceptible cultivar last longer and in panel (b) of this figure it is shown that the non-probing bouts in the resistant cultivar last longer.

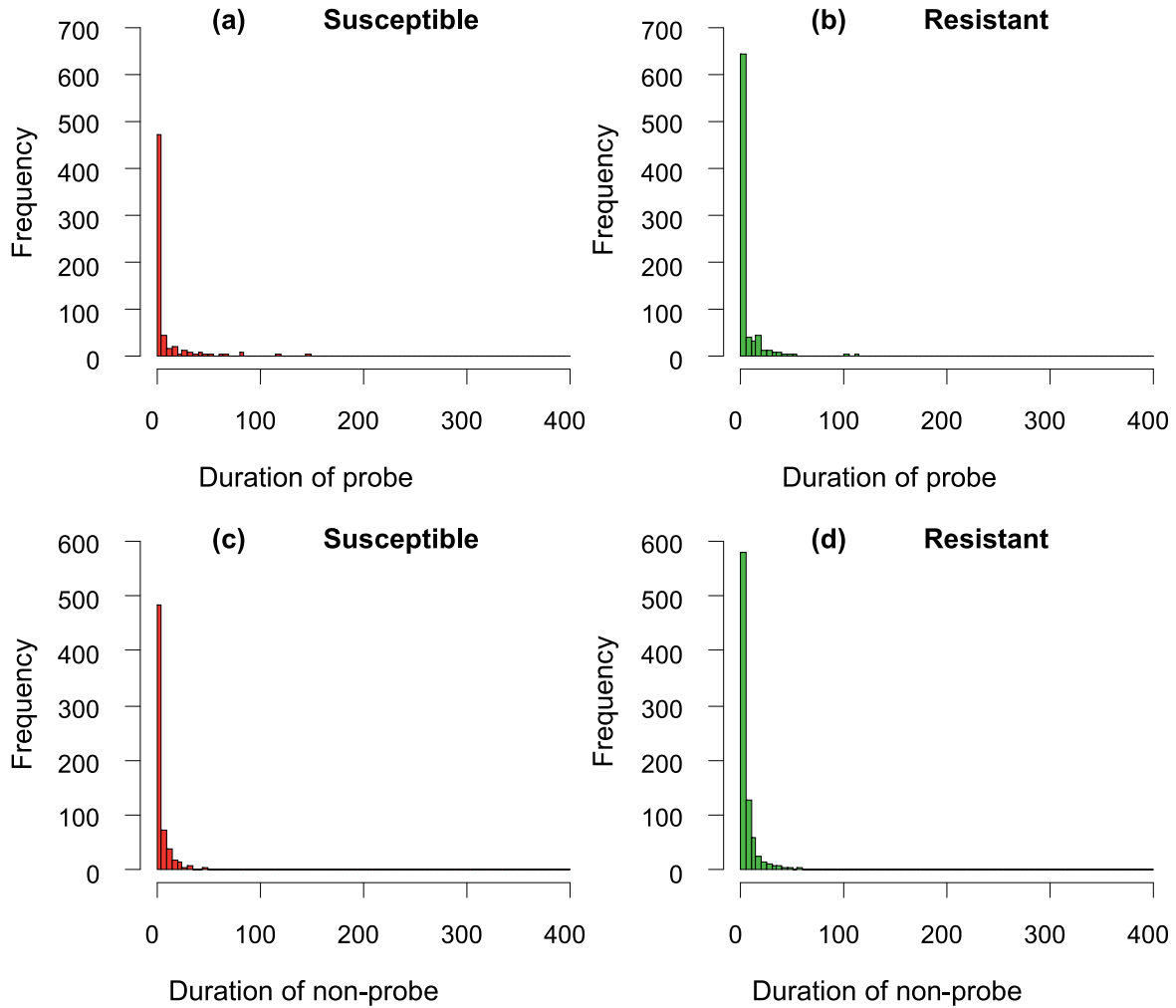


Figure 1. Histograms of probing and non-probing bout lengths are shown for the resistant and susceptible cultivars of lettuce: (a) probes on susceptible cultivar ( $n = 651$ ), (b) probes on resistant cultivar ( $n = 836$ ), (c) non-probes on susceptible cultivar ( $n = 656$ ), and (d) non-probes on resistant cultivar ( $n = 851$ ).

With a Cox proportional hazards model we analysed the effect of the cultivars on probing and non-probing bout lengths. The results are shown in Table 1. The tendency to stop probing bouts on resistant cultivars is 1.21 [ $\exp(\beta_p) = \exp(0.187)$ ] times that on the susceptible cultivar, meaning that on average probing bouts are shorter on resistant cultivars. Mean probe length as defined in Methods and Materials on the susceptible variety is 13.96 and on the resistant variety 7.79 min. The tendency to start probing bouts on resistant cultivars is 0.88 [ $\exp(\beta_N) = \exp(-0.131)$ ] times that on the susceptible cultivar, meaning that on average non-probing bouts last longer on resistant cultivars. Mean non-probe length, as defined in Methods and Materials on the susceptible variety is 6.38 and on the resistant variety 7.84 min.

Table 1. The effect of the cultivar on the probing and non-probing bout lengths is analysed with a Cox proportional hazards model. The baseline hazard is the tendency to, respectively, stop or start probing on the susceptible cultivar. The regression coefficients for probing and non-probing bouts are denoted as  $\beta_P$  and  $\beta_N$ , respectively.

Cultivar	Probing bouts		Non-probing bouts	
	$\beta_P$	P	$\beta_N$	P
Resistant	0.187	<0.001	-0.131	0.012

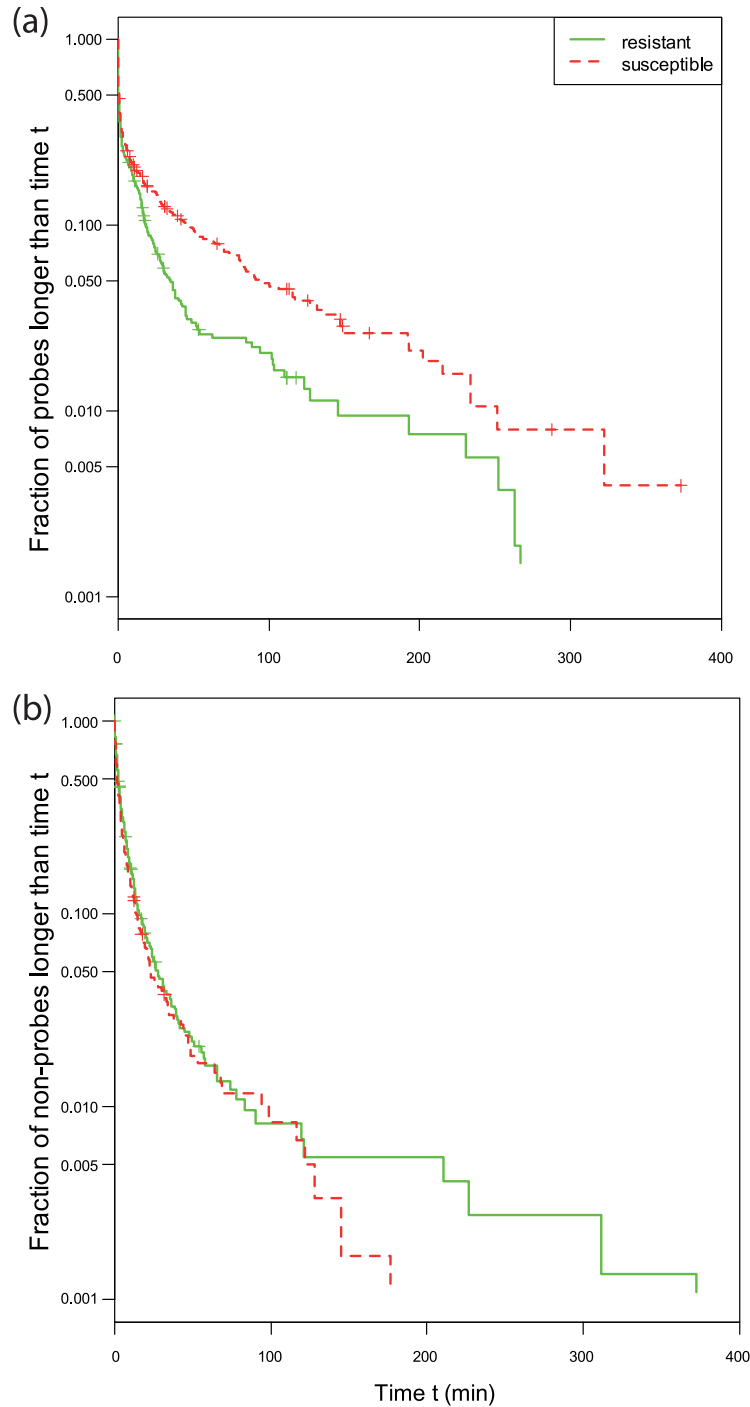


Figure 2. Log-survivor plots of the duration of (a) probing and (b) non-probing bouts.



## DISCUSSION

For both cultivars more than 50% of the probing bouts last less than 1 min. This means that the majority of probes does not involve phloem ingestion, but is rather exploration of plant surface and leaf epidermis or obstruction by elements of these tissues. In addition, more than 50% of non-probing bouts last shorter than 2 min on both cultivars, suggesting that the aphids in general have a strong tendency to start a new probe.

As expected, we found that probing bouts last longer on the susceptible cultivar. This is confirmed by an EPG study with *N. ribisnigri* on these cultivars (ten Broeke *et al.* 2013) and consistent with EPG studies that described the general trend of aphids to perform more and shorter probes on resistant plants (Cook *et al.* 1987, Montllor & Tjallingii 1989, Sauge *et al.* 1998). Rather a new finding is that non-probing bouts last longer on the resistant cultivar. We would expect that a deterrent plant cue would result in a delay of the next probe, but as far as we know, no studies up to now have proven this. The difference between the susceptible and resistant cultivar is, however, more clearly reflected in the duration of the probing bouts than in the duration of the non-probing bouts.

In conclusion, time-to-event techniques prove to be a promising approach for analysing aphid behaviour and identifying resistant plant lines. Instead of using summary statistics such as the sum of probe durations, this method takes the duration of each individual probe into account over the total length of the observation. It thereby delivers more insight into aphid behaviour and orchestrated plant defence mechanisms. Furthermore, it has the advantage that interrupted probing and non-probing bouts at the end of an experiment are correctly included in the analysis. In this study we used a dataset acquired by automated video-tracking, but time-to-event techniques could as well be applied to EPG data. In the future, we will explore these possibilities and incorporate more covariates that might affect probing behaviour, such as experimental design and behavioural factors.

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