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Fasting alters aphid probing behaviour but does not universally increase the transmission rate of non-circulative viruses

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Abstract

A fasting period prior to non-circulative virus acquisition has been shown to increase the rate of transmission by aphids. However, this effect has only been studied for a few virus–vector combinations, and there are contradictory results in the literature as to the role of fasting on virus acquisition. We analysed the influence of fasting on the transmission of three non-circulative viruses, *Cucumber mosaic virus*, *Zucchini yellow mosaic virus* and *Cauliflower mosaic virus*, by two aphid vector species: *Myzus persicae* Sulzer (Hemiptera: Aphididae) and *Aphis gossypii* Glover (Hemiptera: Aphididae). All variables tested, including the virus species and isolate, and the species of aphid, influenced the effect of a fasting period on virus transmission efficiency. Furthermore, when aphids were subjected to an overnight feeding period on a sucrose solution, the fasting effect disappeared and the probing behaviour of these aphids was markedly different to plant-reared aphids. The electrical penetration graph (EPG) technique revealed that fasting altered the probing behaviour of *M. persicae* and *A. gossypii*, with fasted aphids beginning to feed sooner and having a significantly longer first intracellular puncture, measured as a potential drop. Significantly longer sub-phase II-3 of the potential drop and more archlets during this sub-phase were also observed for fasted aphids of both species. However, these behavioural changes were not predictive of increasing virus transmission following a fasting period. The impacts of pre-acquisition fasting on aphid probing behaviour and on the mechanisms of non-circulative virus transmission are discussed.

INTRODUCTION

Fasting aphids prior to an acquisition access period (AAP) has been known for many years to enhance non-persistent virus transmission [1, 2]. Hereafter referred to as the pre-acquisition fasting effect, the phenomenon was first reported to occur when *Myzus persicae* Glover (Hemiptera: Aphididae) was prevented from feeding for as little as five minutes prior to an AAP of cucumber virus 1 (synonym of *Cucumber mosaic virus*, CMV), *Hyoscyamus* virus 3 and potato virus 3 (synonyms of the potyviruses: *Henbane mosaic virus* and *Potato virus Y*, PVY) [1, 2]. Subsequently the pre-acquisition fasting effect on aphid virus transmission has been evaluated and confirmed for several potyviruses [e.g. *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV)] using the same vector, *M. persicae* [3–9]. This effect, however, has not been observed for all potyviruses. For example, a pre-acquisition fasting period did not significantly increase the transmission of *Zucchini yellow mosaic* virus (ZYMV) by *M. persicae*, but did so when *Aphis gossypii* was used as a vector [10]. Similarly, a pre-acquisition fasting period was reported to have no impact on *Cauliflower mosaic virus* (CaMV) transmission by *M. persicae* and *Brevicoryne brassicae* [11, 12].

Two hypotheses have been proposed to explain the pre-acquisition fasting effect on virus transmission. First, compounds from either the plant or aphid saliva could accumulate in the mouthparts of vectors during feeding, and subsequently prevent virus acquisition and retention. Accordingly, such inhibitory compounds would be removed upon fasting [1]. Wang and Pirone [13] reported that fasted *M. persicae* retained virions in their mouthparts more efficiently than non-fasted aphids. This observation was positively correlated with an increase in virus transmission efficiency after a pre-acquisition fasting period. Wang and Pirone also reported that the fasting effect disappeared when *M. persicae* were allowed to feed for an extended

period of time on sucrose solutions. Under these conditions, both fasted and non-fasted aphids displayed similar transmission efficiency to fasted aphids reared on healthy plants [13]. Therefore, these authors suggested that during the fasting period, aphids could release plant-inhibitory compounds previously ingested and retained in their stylets. An alternative hypothesis proposes that fasting alters aphid probing behaviour [14–16]. Instead of having a stereotypical phloem-searching behaviour, fasted aphids were observed to make repetitive, superficial probes that favour the transmission of non-circulative viruses [14, 15]. Detailed monitoring of the probing behaviour of fasted and non-fasted aphids by the electrical penetration graph (EPG) technique [17] on PVY-infected plants during the AAP also revealed differences in the duration of intracellular punctures (commonly termed potential drops: pds) [18]. Fasted aphids produced pds with a significantly longer II-3 sub-phase, a sub-phase correlated to the acquisition of non-circulative viruses. Sub-phase II-3 is the only pd sub-phase in which uptake of cytoplasmic content occurs [19–22]. Similarly, a higher number of archlets during the sub-phase II-3, presumably representing cibarial pumping activity, were also observed in fasted aphids. These observations were correlated with increased PVY transmission [23]. For ZYMV, the length of sub-phase II-3 was also positively correlated with virus acquisition by *M. persicae,* but not with the number of archlets occurring during this sub-phase [22].

The paradigm that pre-acquisition fasting positively impacts non-circulative virus transmission efficiency is based on studies of a few virus–vector combinations [1–3, 5, 9–13]. Nevertheless, aphid fasting prior to transmission experiments is a widely used experimental approach. However, available data do not clearly justify the use of a fasting period prior to virus acquisition. More importantly, the biological mechanism(s) driving the observations associated with aphid fasting and virus transmission are not understood. Furthermore, since Watson's initial report in 1938, little work has been done on cucumoviruses and other non-circulative viruses and this fasting effect, which may be dependent on virus–vector combination and remains to be thoroughly investigated. In summary, a pre-acquisition fasting period is included in the design of most experiments involving non-circulative virus transmission (non-persistent and semi-persistent viruses) and aphid probing behaviour [16, 22, 24–27], despite the fact that the biological role of this artificial experimental detail is unknown, is poorly characterized and that the outcome of its application is not always the same. Because epidemiological models tend to use experimental data on virus transmission rate to simulate pathogen spread, understanding the impact of a fasting period on vector transmission of viruses is of applied relevance.

To address this knowledge gap we studied the effect of pre-acquisition fasting on three non-circulative viruses (cucumoviruses, potyviruses, caulimoviruses) using the aphid vectors *M. persicae* and *A. gossypii*, assessing whether fasting increases transmission rates in all virus–vector combinations. Transmission tests were conducted with fasted and non-fasted aphids, reared on plants or previously fed on sucrose solution, to assess the impact of the plant or plant compounds on virus transmission efficiency. Additionally, aphid behaviour was evaluated by direct observation under a binocular microscope as well as using the EPG technique, to determine the specific probing activities linked to the transmission of non-circulative viruses.

RESULTS

Virus species, isolates and aphid vector species all influence the pre-acquisition fasting effect on transmission efficiency

When aphids were subjected to a fasting period prior to virus acquisition, the impact on virus transmission efficiency was variable among the virus–vector combinations analysed (Fig. 1a–c). Fasted *M. persicae* transmitted all three viruses more efficiently than non-fasted *M. persicae*: CMV-Fny (Fig. 1a; $P<0.001$), CaMV-BJI (Fig. 1b; $P<0.001$) and ZYMV-E17 (Fig. 1c; $P=0.019$). For *A. gossypii*, fasting significantly increased the transmission efficiency of CaMV-BJI (Fig. 1b; $P<0.001$) and ZYMV-E17 (Fig. 1c; $P<0.001$), but not CMV-Fny (Fig. 1a, $P=0.114$).

To determine whether CMV-Fny was representative of the effects of fasting on the transmission of CMV by *A. gossypii,* experiments were conducted using five additional CMV isolates (M6, V698, Ls, Val 24 and B20). These transmission experiments used a slightly different design, five aphids per plant instead of two, and a 5 min AAP instead of 30 s. Under these experimental conditions, fasting only increased the transmission of strain V698 (Fig. 2; $P=0.013$). Fasting did not significantly increase the transmission efficiency of the other CMV isolates: CMV-Fny ($P=0.710$), M6 ($P=0.138$), Ls ($P=0.999$), Val24 ($P=0.999$) and B20 ($P=0.391$).

Pre-acquisition fasting effect disappears when aphids are transitorily fed on sucrose solution prior to virus acquisition

A significant increase in transmission efficiency after fasting was observed for most virus/vector combinations when the aphids were collected directly from plants prior to fasting (Fig. 1), but not when *M. persicae* or *A. gossypii* were given a 16 h feeding period on a 30 % sucrose solution prior to an AAP on virus-infected tissue (Fig. 3). Comparisons of the mean values (Table S1, available in the online version of this article), obtained from three independent experiments, show that fasted *A. gossypii* transmitted the three viruses less efficiently after a feeding period on sucrose solution (Fig. 3), compared to *A. gossypii* reared on zucchini (Fig. 1). This general trend, consistently observed in our data, was also observed for CMV-Fny transmission with non-fasted *A. gossypii* (Table S1).
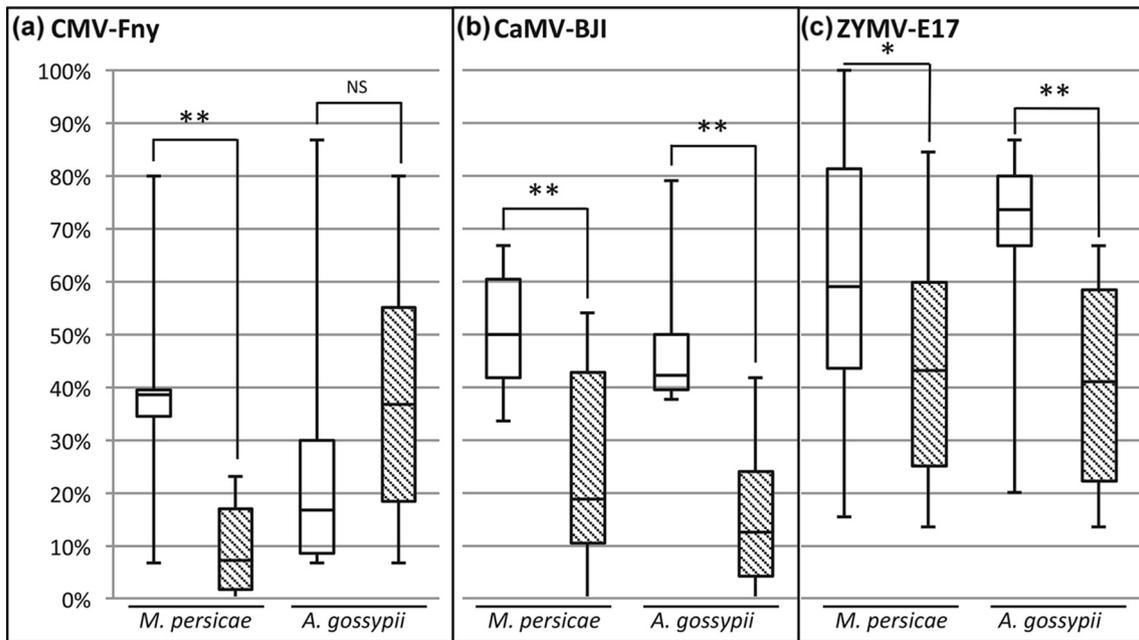
Fig. 1. Pre-acquisition fasting effect on various aphid/virus/plant combinations. Transmission of (a) *Cucumber mosaic virus,* strain Fny; (b) *Cauliflower mosaic virus,* strain BJI and (c) *Zucchini yellow mosaic virus,* strain E17, by *Myzus persicae* and *Aphis gossypii*. Aphids were either fasted for 1 h (white columns) or taken immediately from rearing plants (non-fasted, grey columns) prior to the AAP. Box plots show the median, first and third quartiles of the virus transmission efficiency using two aphids per test plant in three biological replicates of 30 (CMV, ZYMV) to 48 (CaMV) test plants. Two source plants were used for each experiment. Asterisks indicate significant differences between virus transmission efficiencies obtained by either fasted or non-fasted aphids (Chi-square test; *, $P<0.05$; **, $P<0.01$); non-significant: NS.

Effect of fasting on aphid probing behaviour assessed by visual observation

Visual observation was used to compare probing behaviour between cohorts of fasted and non-fasted aphids. We measured: (i) the time taken for aphids to initiate probes once transferred to a source leaf (time to first probe); and (ii) the duration of sustained labium–leaf surface contact, hereafter referred to as the first probing event. These experiments were conducted using virus-infected plants (turnip with CaMV-BJI, zucchini with CMV-Fny or ZYMV-E17). Times to first probing and first feeding event were shorter, regardless of the virus (CMV-Fny, CaMV-BJI and ZYMV-E17), when *M. persicae* reared on eggplant (Fig. 4) and *A. gossypii* reared on zucchini (Fig. 5) were fasted.

When aphids were fed on a 30 % sucrose solution for 16 h, prior to observing their behaviour, the effect was a reduction in the median time to first probe for the fasting treatment relative to the non-fasting treatment, but the differences were not always significant (Figs 4 and 5). The length of the first probing event was reduced by the sucrose feeding for *A. gossypii* and all viruses that we examined; however, the results were variable for *M. persicae*. For *M. persicae* the duration of the first probing event was longer on CMV-infected zucchinis, shorter on CaMV-infected turnips and

the same for fasted aphids compared to non-fasted aphids on ZYMV-infected zucchinis (Fig. 4).

Effect of fasting on aphid probing behaviour assessed by the EPG technique

To better evaluate the behaviour of *A. gossypii* and *M. persicae*, EPG was used to record the type and duration of feeding events of aphids fed on CMV-Fny-infected zucchini plants (Table 1). The time spent by fasted aphids from the start of the EPG recording until the first stylet insertion into the leaf tissue (C waveform) was significantly reduced relatively to non-fasted aphids, from 92.35 to 47.75 s for *A. gossypii* and 38.84 to 19.64 s for *M. persicae* (Table 1). The duration of the C waveform, when aphids secrete the salivary sheath and do not acquire viruses [19, 20, 28], was not significantly different between the treatments for either aphid. The duration of the first pd increased from 7.29 to 9.81 s for *A. gossypii* and from 5.43 to 6.21 s for *M. persicae* for non-fasted and fasted aphids, respectively. Additionally, the duration of the II-3 sub-phase observed in fasted aphids was significantly longer than for non-fasted individuals, from an average of 4.79 and 3.27 s to an average of 7.22 and 4.04 s per aphid for *A. gossypii* and *M. persicae*, respectively (Table 1). The number of archlets during the II-3 sub-phase in fasted aphids was significantly higher than in non-fasted aphids: from an average of 5.35 and 4.34 to an average of
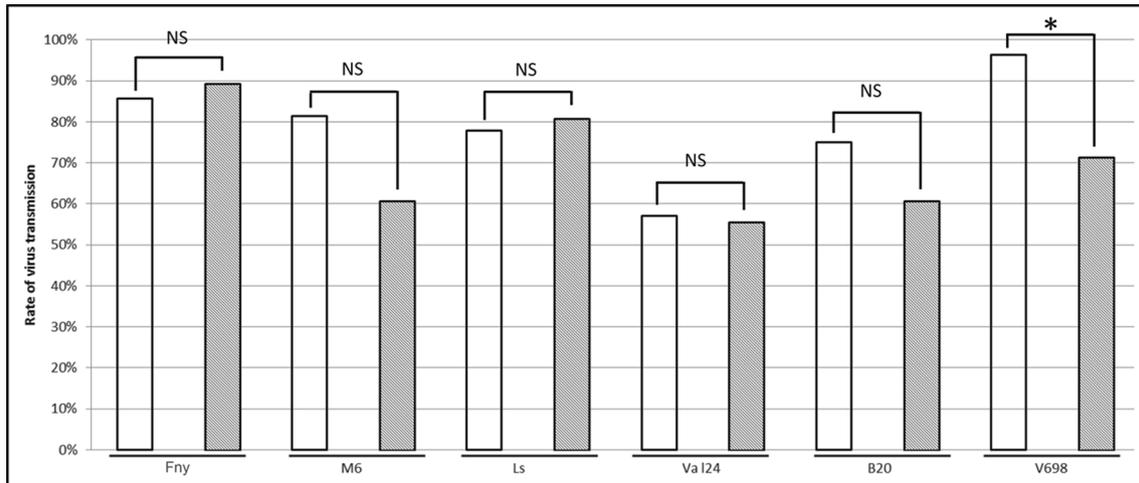
Fig. 2. Transmission of various CMV strains by fasted and non-fasted *A. gossypii*. Transmission of *Cucumber mosaic virus* isolates: Fny, M6, V698, LS, Val24, B20 and V698 by *Aphis gossypii* reared on *Cucumis melo* cv. 'Siglo', using fasted (white columns) or non-fasted (grey columns) aphids prior to the AAP. Box plots show the average virus transmission efficiency using five aphids per test plant in a single replicate of 26–28 test plants. A single-source plant was used for each virus strain. Asterisks indicate significant differences for each fasted/non-fasted pair determined by a Chi-square test (*, $P<0.05$); non-significant: NS.

8.78 and 6.15 per aphid for *A. gossypii* and *M. persicae,* respectively (Table 1). Significant differences in the duration of II-3 sub-phase were observed between fasted aphids of the two species ($P<0.001$), as well as in the number of archlets during II-3 sub-phase ($P<0.001$) (Table 2). However, when non-fasted, the two aphid species did not show
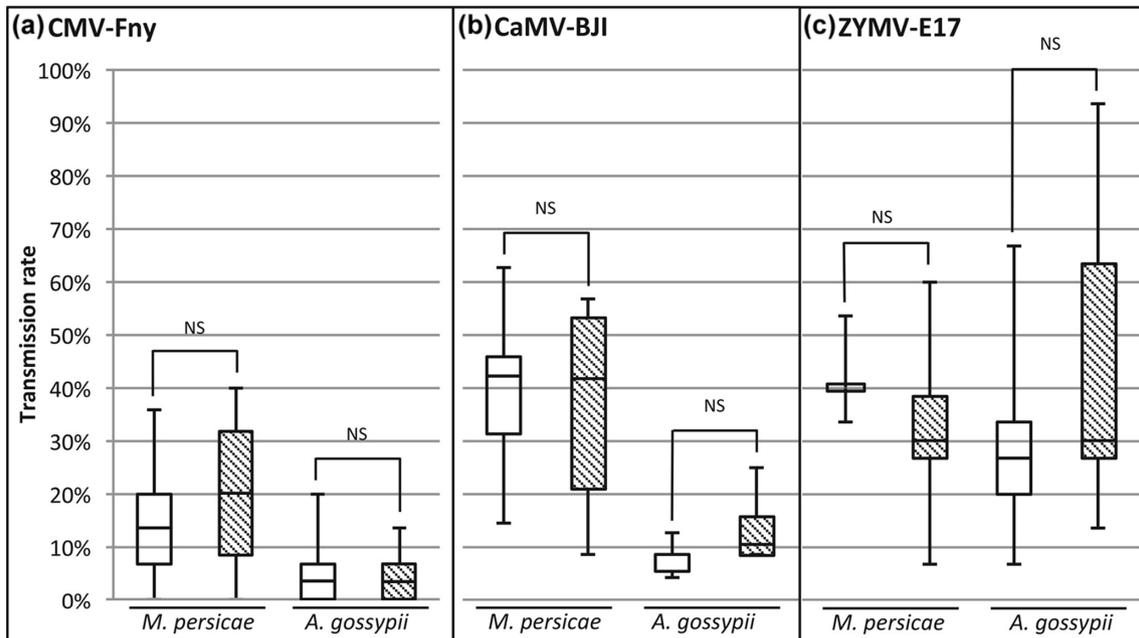
Fig. 3. Pre-acquisition fasting effects on *Myzus persicae* and *Aphis gossypii* reared on sucrose solution. Transmission of *Cucumber mosaic virus* strain Fny, *Cauliflower mosaic virus* strain BJI and *Zucchini yellow mosaic virus* strain E17 from *M. persicae* and *A. gossypii* fed for 16 h on a 30 % sucrose solution and either fasted (white columns) or non-fasted (grey columns) prior to an acquisition access period. Box plots show the median, first and third quartiles of the virus transmission efficiency using two aphids per test plant in three biological replicates of 30 (CMV, ZYMV) to 48 (CaMV) test plants. Two source plants were used for each experiment. No significant differences (NS) were observed for each fasted/non-fasted pair using a Chi-square test for statistical analysis.
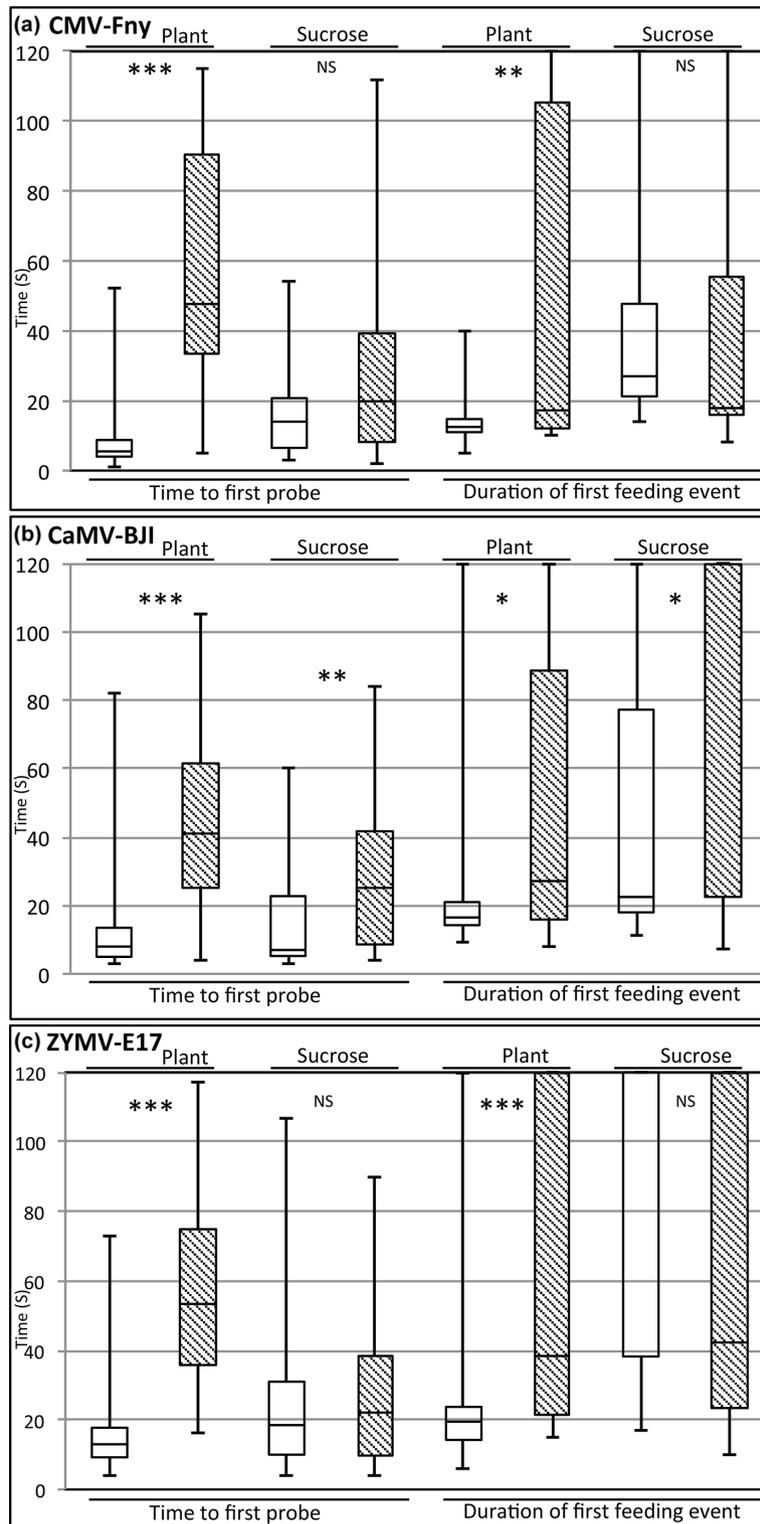
Fig. 4. Probing behaviour of fasted and non-fasted *Myzus persicae* by direct visualization. The time to first probe and the duration of the first feeding event were recorded by direct observation under a dissecting microscope for aphids reared on either eggplant or sucrose solution and allowed to acquire *Cucumber mosaic virus* strain Fny (a), *Cauliflower mosaic virus* strain BJI (b) or *Zucchini yellow mosaic virus* strain E17 (c) during an AAP limited to 120 s. Box plots show the median, first and third quartiles of the duration of the observed aphid probing behaviour. Truncated bars indicate that the median or third quartile of the data was at 120 s. Statistical differences between fasted/non-fasted pairs determined by Mann–Whitney test are indicated using asterisks (·, $P<0.05$; ··, $P<0.01$; ···, $P<0.001$), non significant: NS.
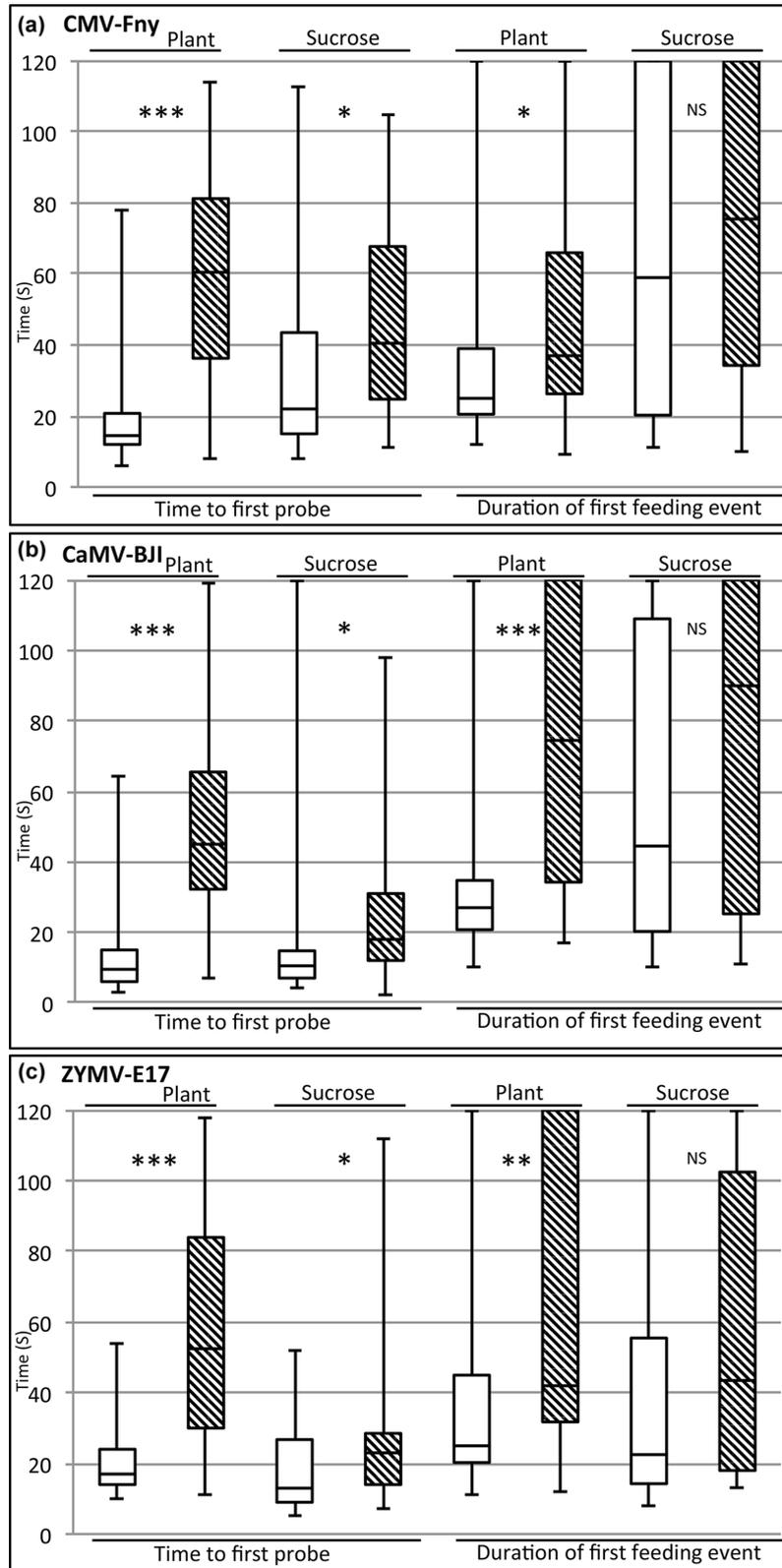
Fig. 5. Probing behaviour of fasted and non-fasted *Aphis gossypii* by direct visualization. Experimental design was the same as that described for *M. persicae* (see Fig. 4).

Table 1. Effect of fasting on the duration of probing behaviour variables, analysed by electrical penetration graph (EPG), of fasted and non-fasted *Myzus persicae* and *Aphis gossypii* on *Cucumber mosaic virus* CMV-Fny-infected *Cucurbita pepo*

Behavioural variables measured (seconds): start of the recording until the beginning of the extracellular stylets pathway, defined as waveform 'C' (Start EPG – C), duration from first C waveform until first intracellular puncture defined as 'pd' (C – pd), total duration of the intracellular puncture, 'pd' (pd duration) and duration of the pd sub-phase II-3 (II-3 duration). Comparison of number of archlets in pd sub-phase II-3 is also shown (No. II-3 archlets).

Behavioral variable	Treatment	*n*	Mean±SE	Min.	Max.	*P* value	Test value
Myzus persicae							
Start EPG – C	Fasted	99	19.64±1.70	1.17	74.90	0.00*	4.22[a]
	Non-fasted	99	38.84±4.13	0.61	203.38		
C – pd	Fasted	99	9.60±0.90	2.15	58.20	0.23	5399[b]
	Non-fasted	99	14.65±2.16	1.65	127.66		
pd duration	Fasted	99	6.21±0.15	3.17	10.01	0.00*	4.04[a]
	Non-fasted	99	5.43±0.18	2.55	11.60		
II-3 Duration	Fasted	99	4.04±0.13	1.23	7.27	0.00*	5.60[a]
	Non-fasted	99	3.27±0.17	0.50	8.93		
No. II-3 archlets	Fasted	99	6.15±0.28	0	12	0.00*	3385[b]
	Non-fasted	99	4.34±0.36	0	15		
Aphis gossypii							
Start EPG – C	Fasted	109	47.75±5.15	2.01	297.60	0.00*	1.64[a]
	Non-fasted	104	92.35±8.11	5.39	299.96		
C – pd	Fasted	109	19.43±4.04	2.88	350.90	0.13	6353[b]
	Non-fasted	104	32.80±4.41	2.93	232.81		
pd duration	Fasted	109	9.81±0.36	2.65	20.67	0.00*	3684[b]
	Non-fasted	104	7.29±0.41	2.65	23.12		
II-3 Duration	Fasted	109	7.22±0.35	0.80	17.15	0.00*	3613[b]
	Non-fasted	104	4.79±0.40	0.42	19.98		
No. II-3 archlets	Fasted	109	8.78±0.50	0	21	0.00*	3605[b]
	Non-fasted	104	5.35±0.61	0	32		

*Significant differences ($P<0.05$) according to a [a]Student's t-test (Gaussian variables) and to a [b]Mann–Whitney U test (Non-Gaussian variables).

such significant differences ($P=0.224$ for II-3 duration; $P=0.896$ for number of archlets) (Table 2).

DISCUSSION

Many variables have been shown to impact the acquisition, retention and inoculation of non-circulative viruses, including virus strain, aphid clone, plant host and previous feeding activities of the aphid vector [26, 29–31]. The fasting of aphids prior to an AAP on virus-infected plants or solutions containing purified virus is generally assumed to significantly increase non-circulative virus transmission [1]. However, the underlying mechanisms explaining this phenomenon are largely unknown. Furthermore, fasting does not appear to increase transmission rate for all virus/aphid combinations (e.g. [26]). We confirmed this observation, that fasting does not increase transmission efficiency in all virus/aphid combinations (i.e. CMV transmission by *A. gossypii*,), and we demonstrate that the fasting effect was not related to any single variable (aphid or virus; Fig. 1). We also showed that, at least for CMV, the occurrence of a fasting effect is also affected by virus isolate. These results challenge the broadly accepted notion that fasting (or the

starvation effect) universally increases non-circulative virus transmission rate.

As previously reported [13], our results confirm the suppression of the fasting effect on virus transmission when aphids are transitorily fed on sucrose solution prior to an AAP on virus-infected plants. Under these conditions, we did not observe any significant differences in transmission rate between fasted and non-fasted aphids for all three viruses tested in this study. These results support the hypothesis that compounds of plant origin present in the stylets of non-fasted aphids interfere with virus uptake and/ or virus retention. Aphid feeding on a sucrose solution also seemed to negatively impact the transmission efficiency of *M. persicae* and *A. gossypii* for the viruses tested when compared to aphids reared on plants, which generally transmitted the three viruses more efficiently. Therefore, the presence of a plant competitor molecule impairing efficient virus uptake and retention at the binding sites in aphid mouthparts alone cannot account for the 'fasting effect' observed.

Fasting aphids prior to an AAP affected the probing behaviour of aphids, with visually observed fasted aphids

Table 2. Comparison of sub-phase II-3 duration (seconds) and number of II-3 archlets between fasted and non-fasted *Aphis gossypii* and *Myzus persicae* when monitoring aphid probing behaviour by EPG on *Cucumber mosaic virus* CMV-Fny-infected *Cucurbita pepo*

Variable	Treatment	*n*	Mean±SE	Min.	Max.	*P* value	U-value
II-3 Duration							
Fasted	*M. persicae*	99	4.04±0.13	1.23	7.27	0.00*	8504
	A. gossypii	109	7.22±0.35	0.80	17.25		
Non-Fasted	*M. persicae*	99	3.27±0.17	0.50	8.93	0.22	5656
	A. gossypii	104	4.79±0.40	0.42	19.98		
II-3 archlets							
Fasted	*M. persicae*	99	6.15±0.28	0	12	0.00*	3572
	A. gossypii	109	8.78±0.50	0	21		
Non-Fasted	*M. persicae*	99	4.34±0.36	0	15	0.90	5094
	A. gossypii	104	5.35±0.61	0	32		

*Significant differences ($P<0.05$) according to a Mann–Whitney U test.

beginning to feed sooner, and producing shorter first probing events. While not significant in all comparisons, these variations among treatments support the hypothesis that changes in aphid probing behaviour may explain the fasting effect on non-circulative virus transmission rate [14–16]. We attempted to identify the underlying causes of the fasting effect on transmission by comparing the probing behaviour of *M. persicae* and *A. gossypii* monitored by EPG during acquisition of CMV-Fny. Similar to a previous report [16], fasting affected both aphid species in a similar manner, causing insects to initiate probing faster and make longer first pds, have longer II-3 sub-phase, as well as a higher number of archlets on sub-phase II-3. During that specific pd sub-phase, it has been suggested that aphids ingest cytosol contents from punctured cells by active uptake using the cibarium as a pump. Therefore, aphids showing this behaviour are likely to acquire non-circulative viruses more efficiently [19, 20]. The EPG recording indicated that fasted *M. persicae* spent only 9.6 s from the start of C waveforms until the first potential drop was observed. Therefore, during the given 30 s AAP, an aphid would be able to do more than one pd and acquire the virus in several cells (average of one pd is 6.21 s in length). Such a change in behaviour could increase the chances of CMV acquisition or retention and therefore transmission during fixed duration AAPs. However, these EPG variables (longer first pd, longer II-3 sub-phase) of fasted aphids did not always correlate with increased transmission rate. Fasted *A. gossypii* transmitted CMV with less efficiency than fasted *M. persicae*, despite a higher number of archlets in II-3 sub-phase when monitoring the aphid probing behaviour. The overall longer duration of the C waveform meant that fasted and non-fasted aphids likely produced only a single pd during the AAP of 30 s, potentially explaining why fasting did not significantly increase CMV transmission.

Wang *et al.* [32] suggested possible differences between aphid vector species when comparing the transmission of TEV and TuMV by *M. persicae* and *Myzus ascalonicus* van der Goot

(Hemiptera: Aphididae). The probing behaviour of fasted individuals of both species used in this study was similar (same occurrence of potential drops and duration of pd sub-phases). However, differences in transmission efficiency were observed. Other variables may explain the contrasting effect of fasting observed for different virus/vector combinations. For example, virus infection modifies the composition and/or the concentration of volatiles emitted by plants, some of which act as aphid attractants [33]. At this stage, we cannot rule out the possibility that non-fasted and fasted aphids evaluate the attractiveness for infected and non-infected plants differently. Regarding the insect partner, differences in the chemical composition of the inner surface of aphid stylets [34] and/or other physiological aspects, such as flushing out of virions by egested saliva and the composition of the saliva itself, might differ between *M. persicae* and *A. gossypii*. Interestingly, differences in the composition of aphid saliva are supported by the observation of similar probing behaviours by *A. gossypii* and *M. persicae* on a melon accession carrying the *Vat* resistance gene [35, 36]; only *A. gossypii* triggered a hypersensitive response after superficial intracellular stylet punctures. Additional studies will be required to better understand how fasting impacts the transmission of non-circulative viruses, and to determine which factors in addition to aphid probing behaviour are involved in the transmission success. Such knowledge would undoubtedly bring novel information on plant–virus–insect interactions.

METHODS

Virus maintenance and aphid colonies

Isolates of CMV (Fny, M6, V698, Ls, Val 24 and B20) and ZYMV (E17) were maintained on *Cucurbita pepo* cultivar (cv.) 'Précoce Maraîchère'. CaMV (isolate Cabb BJI) was maintained on *Brassica rapa* cv. 'Just Right'. Isolates were mechanically passaged weekly in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 % (wt/vol) sodium sulfite and 1 % (wt/vol) Celite. The clone of *M. persicae* used in this study was collected in the South of France over 30 years ago

[37]. *M. persicae* were reared on *Solanum melongena* cv. 'Barbentane'. *A. gossypii*, clone NM1 [38] were reared on *C. pepo* cv. 'Précoce Maraîchère' and on *Cucumis melo* cv. 'Siglo'. All colonies were maintained in a growth chamber at a 23/18 °C (day/night) and a photoperiod of 16/8 h (day night^{-1}) ensuring clonal reproduction.

Transmission tests with fasted and non-fasted aphids reared on plants or on sucrose solutions

Apterous adults *M. persicae* reared on eggplant cv. 'Barbentane', and *A. gossypii* reared on *C. pepo* cv. 'Précoce Maraîchère', were collected from plants and either isolated for one hour in glass vials (fasted aphids) or transferred directly from the rearing plant on a virus-infected source leaf (non-fasted aphids). Alternatively, aphids collected on rearing plants were allowed to feed on 30 % sucrose solution held between two layers of stretched Parafilm (Neenah, WI, USA) for 16 h and either fasted for 1 h or directly used in transmission tests. Both fasted and non-fasted aphids were moved by paintbrush to upper, fully expanded virus-infected leaves. The length of time to first probe (time taken by an aphid placed on a leaf surface to show sustained contact between its rostrum and the leaf surface) and the duration of the first probing event (time spent at the first probing site until the aphid has withdrawn its rostrum from the leaf surface) were measured by direct observation of the insects under a dissecting microscope during the AAP. Aphids were considered to have successfully completed an AAP when they were observed to have sustained contact between the rostrum of the aphid and the infected source leaf for 15 to 30 s. Aphids that did not show sustained labium–leaf surface contact within the first two minutes were discarded. Two aphids were then transferred to each zucchini (for CMV and ZYMV) or turnip (for CaMV) test plant and allowed an inoculation access period (IAP) of two hours, before the aphids were killed by an insecticidal treatment. Symptoms of virus infection were recorded 21 days later by visual inspection as previously reported [39–41]. Three independent biological replicates were done for each virus–vector combination.

To assess whether a fasting effect was observed for different viral strains in a single virus species, transmission tests were performed on six CMV isolates – Fny, M6, V698, Ls, Val 24 and B20 – using *A. gossypii* reared on *C. melo* cv. 'Siglo'. In these series of experiments, five aphids per test plant were allowed a 5 min AAP on CMV-infected plants, followed by 2 h IAP before insecticide treatment. Transmission efficiency was calculated by the number of plants expressing virus symptoms 21 days post inoculation divided by the total number of test plants.

Electrical penetration graph recording

To understand aphid probing behaviour, an EPG device (Giga-8; EPG-Systems) connected to a USB AD card (DI-710; DATAQ Instruments) and a laptop PC was used to record electronic signatures that were analysed using Stylet +Software (EPG Systems, Wageningen, The Netherlands;

[17]). Newly emerged non-viruliferous *M. persicae* adults reared on eggplant cv. 'Barbentane', as well as *A. gossypii* reared on *C. melo* cv. 'Siglo', were used for the experiments. Aphids were individually immobilized at the edge of a pipette tip using a vacuum pump and then attached by a gold wire to the dorsum using a small water-based silver paint (EPG Systems, Wageningen, The Netherlands). After wiring, some aphids were suspended in the air by the wire and not allowed to feed (fasted), while others were placed directly on either a healthy melon leaf (*A. gossypii*) or a healthy eggplant leaf (*M. persicae*) and allowed to feed (non-fasted). After 1 h, both fasted and non-fasted aphids were connected to the EPG device and placed on a CMV-Fny-infected zucchini plant until a single intracellular puncture (pd) was observed. Aphids that did not start to probe within 5 min were discarded. Once the pd was observed, recordings were manually ended by softly touching the aphids with a brush. Duration of some EPG waveforms, previously described for aphids [28], and relevant for non-circulative virus transmission [42], were considered as variables to compare the probing behaviour of fasted and non-fasted aphids: (i) time spent from start of the EPG recording until first probe (Start – C; C waveform reflecting the apoplastic stylets pathway); (ii) time from first probe until intracellular puncture (C – pd); (iii) duration of intra-cellular puncture (pd duration); and (iv) time spent from the start of the recording until the intracellular puncture (Start – pd). Finally, among the three distinct sub-phases occurring during an intracellular puncture (II-1, II-2 and II-3), the duration of the II-3 sub-phase of the intracellular puncture, as well as the number of archlets within this sub-phase were quantified. Longer duration of this sub-phase and thus larger number of archlets, have been positively correlated with acquisition efficiency of non-circulative viruses [19, 20].

Statistical analysis

The analysis of the duration of the different behavioural variables studied by either EPG recording or binocular microscope was conducted using SPSS v.22 software package (IBM SPSS statistics, 2013). Behavioural variables were transformed by either ln (x+1) or sqrt (x+1) prior to analysis and later checked for normality using Shapiro–Wilk test. Mean comparison between groups of fasted and non-fasted aphids that followed a Gaussian distribution was analysed by Student's *t*-test to detect any significant difference (α=0.05). When behavioural variables did not follow a Gaussian distribution, non-parametric Mann–Whitney *U*-test test was then applied. On the other hand, virus transmission rates obtained by either fasted or non-fasted aphids were compared performing a Chi-square test or a Fisher's Exact test when expected values were lower than 5, using Stat View 4.0 statistical package (α=0.05) [43].

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