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# The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny

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The phloem-sucking psyllid *Cacopsylla picta* plays an important role in transmitting the bacterium '*Candidatus* Phytoplasma mali', the agent associated with apple proliferation disease. The psyllid can ingest '*Ca.* Phytoplasma mali' from infected apple trees and spread the bacterium by subsequently feeding on uninfected trees. Until now, this has been the most important method of '*Ca.* Phytoplasma mali' transmission. The aim of this study was to investigate whether infected *C. picta* are able to transmit '*Ca.* Phytoplasma mali' directly to their progeny. This method of transmission would allow the bacteria to bypass a time-consuming reproductive cycle in the host plant. Furthermore, this would cause a high number of infected $F_1$ individuals in the vector population. To address this question, eggs, nymphs and adults derived from infected overwintering adults of *C. picta* were reared on non-infected apple saplings and subsequently tested for the presence of '*Ca.* Phytoplasma mali'. In this study it was shown for the first time that infected *C. picta* individuals transmit '*Ca.* Phytoplasma mali' to their eggs, nymphs and $F_1$ adults, thus providing the basis for a more detailed understanding of '*Ca.* Phytoplasma mali' transmission by *C. picta*.

*Keywords:* apple proliferation, epidemiology, insect vectors, phytoplasma & relatives, psyllids, transovarial transmission

## Introduction

'*Candidatus* Phytoplasma mali' is the agent associated with apple proliferation (AP), a major threat in several apple-growing regions (Bertaccini *et al.*, 2014). The most important symptom is the production of small, tasteless and colourless fruits, which leads to large financial losses in affected apple-growing areas (Bertamini *et al.*, 2002; Tedeschi *et al.*, 2003; Bertaccini *et al.*, 2014).

Phytoplasmas are transmitted by insect vectors belonging to the taxonomic groups Cicadellidae, Fulgoromorpha and Psyllidae (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2014), and can additionally be transmitted by natural root grafts (Baric *et al.*, 2008). In northern Italy the two phloem-feeding psyllids *Cacopsylla picta* (Hemiptera: Psyllidae) and *Cacopsylla melanoneura* are vectors of '*Ca.* Phytoplasma mali' (Frisinghelli *et al.*, 2000; Tedeschi *et al.*, 2002; Carraro *et al.*, 2008), while in Germany only *C. picta* was found to be able to transmit the pathogen (Mayer *et al.*, 2009; Jarausch *et al.*, 2011).

Studies conducted in Trentino and South Tyrol (northern Italy) show a higher transmission efficiency for *C. picta* compared to *C. melanoneura* (Mattedi *et al.*, 2008). From the end of March to April, remigrant individuals of *C. picta* migrate from their overwintering shelter plants into apple orchards for reproduction and feeding (Mattedi *et al.*, 2008). *Cacopsylla picta* accomplishes one generation per year and the development from eggs to $F_1$ adults involves five larval instars. *Cacopsylla picta* offspring (emigrants) leave the apple orchards in July and migrate to the overwintering shelter plants (Mattedi *et al.*, 2008).

The life cycle of the bacterium '*Ca.* Phytoplasma mali' is strongly connected to its insect vector and the host plant (Mayer *et al.*, 2008a,b; Bertaccini *et al.*, 2014). The insect vectors acquire the AP phytoplasma by ingesting plant sap from the phloem of infected apple trees (Pedrazzoli *et al.*, 2007; Weintraub, 2007; Mattedi *et al.*, 2008). After ingestion, the phytoplasma move through the food canal of the stylet and invade different cellular tissues of the insects. The bacteria multiply in secretory salivary glands and are translocated into a new plant via the saliva when the infected insect feeds (Hogenhout *et al.*, 2008b).

Currently treatments preventing the spread of transmitting insects and the uprooting of infected trees are the only ways to limit disease spread in affected regions (Baric *et al.*, 2010).

Aside from the acquisition of infected phloem sap by ingestion, another putative way for phytoplasma spread would be a transovarial or 'vertical' pathogen transmission. This type of transmission is well known in plant virus insect vectors (Hogenhout *et al.*, 2008a).

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Plant Pathology

Transovarial transmission of plant viruses occurs only in the group of viruses that persist and replicate inside their insect vectors (Hogenhout *et al.*, 2008a; Huo *et al.*, 2014). However, until now, transovarial transmission was reported only in 4% of phytoplasma-transmitting insect vectors (Arismendi *et al.*, 2015). Vertical transmission was found in the leafhopper species *Scaphoideus titanus*, the vector of aster yellows phytoplasma (Alma *et al.*, 1997), in *Hishimonoides sellatiformis*, the vector of mulberry dwarf phytoplasma (Kawakita *et al.*, 2000), and in *Matsumuratettix hiroglyphicus*, the vector of sugarcane white leaf phytoplasma (Hanboonsong *et al.*, 2002). Tedeschi *et al.* (2006) showed that females of *Cacopsylla pruni* infected by '*Candidatus* Phytoplasma prunorum' are able to vertically transmit the pathogen and demonstrated infectivity of transovarially infected $F_1$ individuals. However, those authors could not show transovarial transmission of '*Ca.* Phytoplasma mali' in *C. melanoneura*. The ability of *C. picta* to vertically transmit '*Ca.* Phytoplasma mali' was hypothesized (Tedeschi *et al.*, 2006) but has never been experimentally addressed.

Thus, the aim of this study was to test whether '*Ca.* Phytoplasma mali'-infected *C. picta* are able to vertically transmit the pathogen to their progeny. Specifically, the study aimed to determine whether the number of $F_1$ individuals deriving from infected or uninfected parental females differs and whether the phytoplasma titre changes between developmental stages of the insect. Additionally, a rearing-feeding-oviposition method was established to study the influence of a potential phytoplasma acquisition on the puncture site of parental insects.

## Materials and methods

### Insects

Overwintering adults of *C. picta* were collected in April 2016 using the beating tray method (Müther & Vogt, 2003) in two abandoned apple orchards in Valsugana Valley (Trentino, Italy). The collected living individuals were isolated in glass collection tubes, anaesthetized on ice and morphologically characterized using identification keys of Ossiannilsson (1992). The collected winter generation (remigrants) of *C. picta* will henceforth be referred to as 'parental generation', and the reared summer generation (emigrants) as '$F_1$ generation'.

### Insect rearing

Insects from the natural populations were reared at Laimburg Research Centre (Laimburg) and at Fondazione Edmund Mach (FEM), Italy. A total number of 37 couples (27 at Laimburg and 10 at FEM) of parental females and males of *C. picta* were released into single net-cages under controlled conditions (15–25 °C, natural light and 70–100% relative humidity), on recently (same year) grown apple bud grafts (cv. Golden Delicious on M9 rootstock). Each couple was released on an individual plant. Additionally, 14 parental females (12 at Laimburg, 2 at FEM) were single-caged on grafts. Cages were monitored every day for vitality of parental individuals and the presence of eggs. After egg laying, parental insects were collected, frozen at −80 °C, and subsequently analysed by PCR for detection of '*Ca.* Phytoplasma mali' DNA (described below). Based on the PCR results of *C. picta* parental individuals, plants with eggs were selected and divided into three groups (Fig. 1; Table 1): plants with progeny from an infected parental female; plants with progeny from an uninfected female and an infected parental male (control group 1); and plants with progeny from both uninfected female and male parental individuals (control group 2).

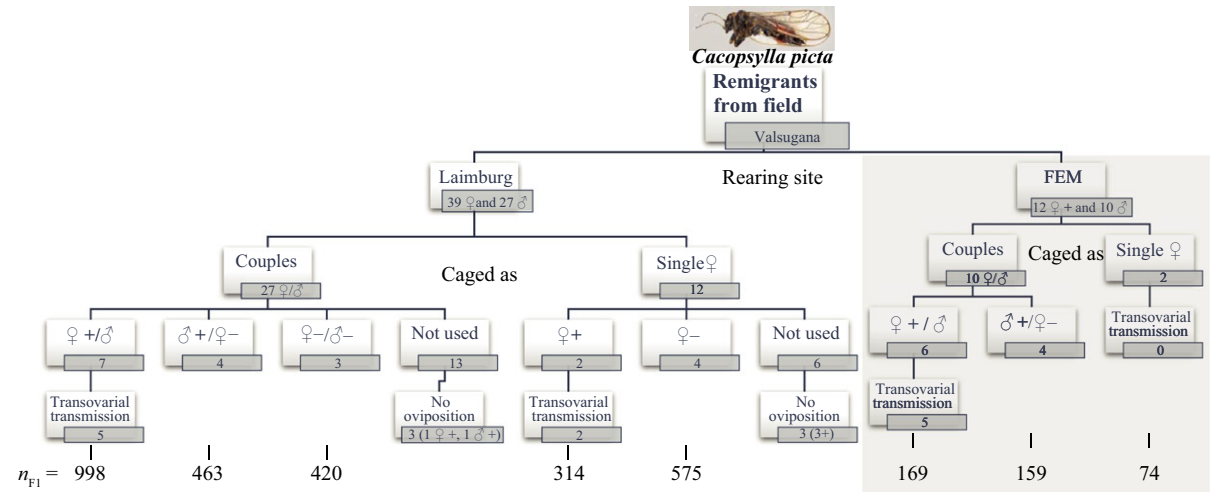


Figure 1 Schematic overview of experimental design. Remigrant *Cacopsylla picta* individuals collected in orchards in Valsugana Valley (Trentino Province, Italy) were caged as couples or single individuals (numbers are given in grey boxes). Rearing experiments were performed at two different sites: at Laimburg Research Centre (Laimburg) and at Fondazione Edmund Mach (FEM). Remigrants were sorted according to their state of infection with '*Candidatus* Phytoplasma mali' as follows: infected females (♀ +), infected males (♂ +), uninfected females (♀ −) and uninfected males (♂ −). Numbers of analysed $F_1$ individuals ($n_{F1}$) of the respective parental groups are mentioned underneath the chart. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1 Total number of analysed *Cacopsylla picta* parental and $F_1$ individuals

| Infection state of remigrants | No. of parental females[a] | Developmental stage ($F_1$)[b] | | | | | | | Total ($F_1$) |
|---|---|---|---|---|---|---|---|---|---|
| | | Egg | L1 | L2 | L3 | L4 | L5 | Adult | |
| Infected parental female | 17 | 389 | 428 | 131 | 109 | 57 | 268 | 173 | 1555 |
| Control group 1, uninfected parental female, infected parental male | 8 | 73 | 191 | 33 | 86 | 85 | 91 | 63 | 622 |
| Control group 2, uninfected parental individuals | 7 | 244 | 320 | 7 | 93 | 69 | 135 | 127 | 995 |
| Total | 32 | 706 | 939 | 171 | 288 | 211 | 494 | 363 | 3172 |

[a]Remigrant parental individuals were collected in apple orchards in Valsugana Valley and released onto single net-caged apple graftings for oviposition at Laimburg Research Centre or at Fondazione Edmund Mach, Italy.
[b]$F_1$ individuals were collected from the apple graftings. L1 to L5, nymphal instars.

One or two leaves with eggs were taken from the branches of each group. Using titanium needles and a stereo-zoom microscope (Zeiss Axiozoom V16), all eggs were removed from leaves and pooled in batches of five. Developmental stages of *C. picta* were documented with ZEN PRO microscope software. In the following weeks, assorted samples of each instar-stage (first instars in batches of five, from second instars to $F_1$ adults as single insects) were collected and frozen at −80 °C. The remaining insects were left on the branches to complete development until the adult stage, when they were collected and frozen at −80 °C.

In total, the study group consisted of 17 infected parental females captured in orchards located in Valsugana Valley (Trentino) and reared in single-net cages (nine at Laimburg, eight at FEM) and a total of 1555 $F_1$ individuals were analysed. Control group 1 consisted of eight uninfected females (four at Laimburg, four at FEM) caged with an infected parental male. The resulting 622 $F_1$ individuals were analysed, as well, to study the importance of a potential passive impact of an infected individual on the transmission rate, by locally providing bacterial inoculum to the uninfected host plant. Control group 2 comprised seven uninfected parental females and males, all reared at Laimburg, with 995 $F_1$ individuals serving as a negative control (Fig. 1; Table 1).

At the end of the experiment, phloem tissue was isolated from root samples of plants where eggs had developed and was stored at −80 °C for subsequent DNA extraction to assess the presence or absence of '*Ca.* Phytoplasma mali'.

## DNA extraction and PCR analysis

Isolation of DNA from insects was performed using the DNeasy Blood and Tissue extraction kit (QIAGEN). DNA from remigrants reared at FEM was extracted with the Nucleo Spin tissue kit (Macherey-Nagel). DNA of each insect, egg or first instar batch (five eggs or first instar nymphs were pooled prior to DNA purification) was eluted with 100 µL TE elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0).

Genomic DNA from plants was extracted according to the manufacturer's instructions with a DNeasy Plant Mini kit (QIAGEN) including the following optimization step: plant material (100 mg) was disrupted by addition of 400 µL buffer AP1, for 3 min in a TissueLyser II (QIAGEN). To the disrupted plant material, 4 µL RNase A were added and the mix was incubated for at least 30 min in a water bath at 65 °C. DNA was eluted two times with 50 µL TE buffer as mentioned above.

'*Candidatus* Phytoplasma mali' DNA was detected by SYBR Green real-time PCR with primers rpAP15f-mod and rpAP15r3, as described in Monti *et al.* (2013), targeting the ribosomal protein gene *rpl22*. A 2 µL sample of template DNA was mixed with 5 µL of 2× SYBR FAST qPCR Kit Master Mix (Kapa Biosystems), 2.5 µL nuclease-free water and 0.25 µL each of forward and reverse primer (10 µM). The cycling conditions were as follows: initial denaturation at 95 °C for 20 s; 35 cycles of 95 °C for 3 s and 60 °C for 30 s; and a melting curve ramp from 65 to 95 °C at increments of 0.5 °C every 5 s (CFX384 Touch Real-Time PCR Detection System; Bio-Rad). All insects were tested individually and each sample was tested in triplicate in three independent PCR runs.

Phytoplasma PCR detection limits were carefully determined using a four-point 10-fold dilution series (diluted in TE buffer; $6.5 \times 10^4$–$6.5 \times 10^1$ DNA copies per sample) of the plasmid pJET1.2-*rpl22* containing the subcloned '*Ca.* Phytoplasma mali' *rpl22* PCR amplicon. The dilution series was included in every real-time PCR run. Samples with a mean quantification cycle ($C_q$) value lower than 30 and a melting curve peak similar to the positive control were considered '*Ca.* Phytoplasma mali'-positive. The phytoplasma titre was quantified based on the four-point plasmid standard curve analysed in parallel with the samples in each PCR run.

As a control of DNA integrity and to normalize amounts of phytoplasma, a region of the single-copy *wingless* (*wg*) gene (Brower & DeSalle, 1998) of *C. picta* was amplified, in parallel to the amplification of the *rpl22* fragment, with primers specific for *C. picta* and other psyllid species, qPSY-WG-F (5′-TC ACGGGCGGCAATG-3′) and qPSY-WG-R (5′-CCCACAGCA CATCAGATCACA-3′). The PCR was performed as described above with 0.25 µM each of the *wg*-specific forward and reverse primer; included in each PCR run was a dilution series ($7.3 \times 10^5$–$7.3 \times 10^2$ DNA copies per sample) of the plasmid pJET1.2-*wg* containing the subcloned *wg* gene PCR amplicon. The dilution series and standard curves were prepared as previously described for the pJET1.2-*rpl22* plasmid. Phytoplasma concentration was then calculated in relation to the *wg* gene.

Phytoplasma concentration was quantified within the range of the four-point standard dilution series and samples above the range were diluted in elution buffer and reanalysed if necessary. Samples with a mean $C_q$ value above 30 were considered '*Ca.* Phytoplasma mali'-negative and detection limits could be verified to be comparable as described in Monti *et al.* (2013). Because of batch analysis, quantification of '*Ca.* Phytoplasma mali' in egg or first instar batches was not possible.

Threshold calculation and data analysis was performed using CFX MANAGER software (Bio-Rad), considering only runs with a PCR efficiency between 95% and 105% and a coefficient of determination ($R^2$) ≥0.99. Three non-template controls (NTC, nuclease-free water) were performed together with each PCR run. Plant material was analysed with the same *rpl22*-based SYBR approach as described above. DNA integrity from plant samples was verified by real-time PCR with a probe for a chloroplast gene as described in Baric & Dalla-Via (2004).

Performance of qPCR for quantification of the *wg* gene was in compliance with the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al.*, 2009; Table S1).

### Confirmation of psyllid species identification by PCR-RFLP

Morphological identification of live psyllid specimens is cumbersome and less precise than identification of dead insects. Certain important morphological characteristics, such as wings or terminalia, cannot be properly inspected in detail without harming the insect. To verify the accuracy of morphological psyllid species identification of living parental insects after finalizing the experiments, DNA from all parental individuals was analysed by restriction fragment length polymorphism (RFLP) according to Oettl & Schlink (2015). Only insects morphologically and genetically identified as *C. picta* were considered in this study.

### Statistical analysis

For comparison of phytoplasma titre increase in instars, a one-way ANOVA with a Tukey post hoc test was applied, while in all other comparisons, Student's *t*-test was used. All data were statistically analysed using GRAPHPAD PRISM v. 7.0.

## Results

### Infected *C. picta* females produce infected progeny

Phytoplasma were detected in 30.2% (average per parental female) of the tested egg batches. The percentage detected increased with each developmental stage and, finally, 99.1% of the $F_1$ adults (average rate per parental female) were found to contain phytoplasma (Fig. 2). Phytoplasma concentration in eggs and first instar batches was very low but unambiguous as characterized by a $C_q < 30$ and a specific amplicon melting peak.

Phytoplasma concentration was determined as the ratio of *rpl22*:*wg* gene copies. The phytoplasma titre increased exponentially from second instar nymphs to the $F_1$ adult stage. A significant increase was detected between the titre of fourth and fifth instar nymphs ($P = 0.0249$) and between fifth instar nymphs and $F_1$ adults ($P < 0.0001$). $F_1$ adults contained 100-fold more phytoplasma than second instars (Fig. 3) and comparable concentrations to those of parental females (Fig. 4).

Five out of 17 infected parental females of the study group did not transmit the phytoplasma to their progeny at all. Parental females that did not transmit phytoplasma to their progeny had a significantly lower phytoplasma titre

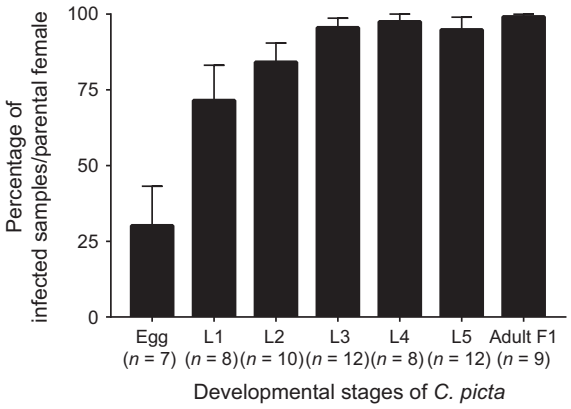


Figure 2 The percentage of *Cacopsylla picta* infected with '*Candidatus* Phytoplasma mali' at different stages of insect development. Columns show the average percentage of egg batches, L1 nymph batches and L2 nymph – $F_1$ adults produced by infected parental females ($n$ = total number of infected parental females), in which phytoplasma was detectable. Bars show the standard error of the mean.

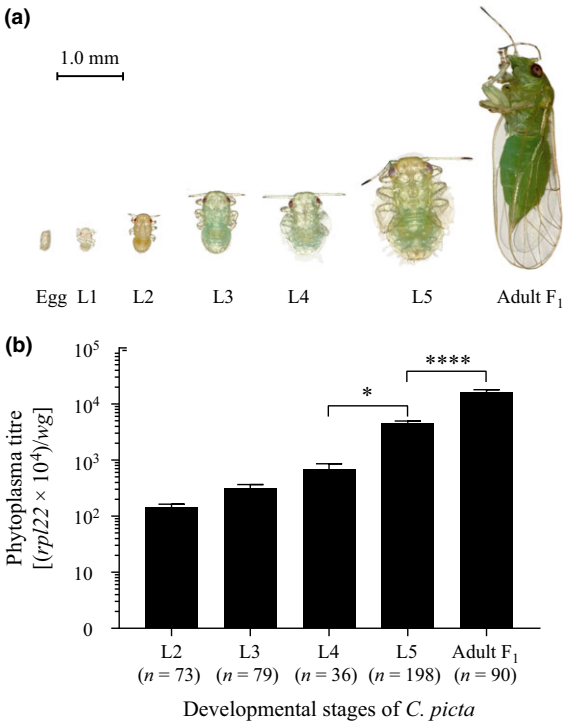


Figure 3 Developmental stages of *Cacopsylla picta* (a) and exponential increase in titre of '*Candidatus* Phytoplasma mali' during insect development (b). (a) Egg, nymphal instars (L1–L5) and $F_1$ adult of *C. picta*. (b) Phytoplasma titre at different stages of infected *C. picta* is given as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Vertical bars show the standard error of the mean. Statistical differences are indicated by $*P \leq 0.05$, $****P \leq 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com].

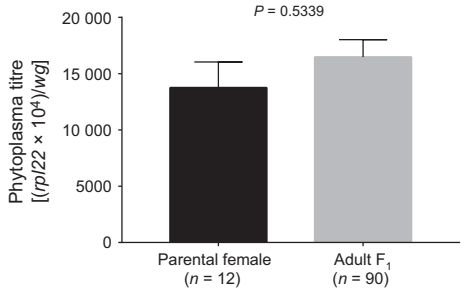


Figure 4 The titre of '*Candidatus* Phytoplasma mali' in *Cacopsylla picta* parental females and $F_1$ adults, shown as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Error bars indicate the standard error of the mean for each experimental group. There is no significant difference between the two groups ($P = 0.5339 > 0.05$, $t = 0.6242$, d.f. = 100).

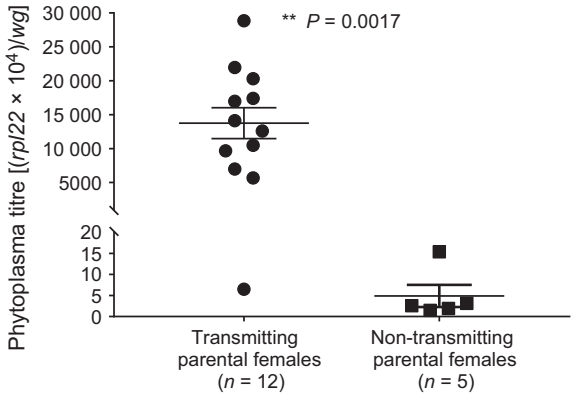


Figure 5 The correlation between transovarial transmission and the bacterial load of parental females. Infected *Cacopsylla picta* parental females were clustered into two groups based on their ability to transovarially transmit '*Candidatus* Phytoplasma mali'. Bacterial load was measured by the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Each dot represents the bacterial load of a *C. picta* individual that was able to transmit '*Ca.* Phytoplasma mali' to its progeny. Each square shows the bacterial load of a *C. picta* female that did not transmit '*Ca.* Phytoplasma mali' to its progeny. Horizontal bars show the mean, while vertical bars show the standard error of the mean of each group. Statistical differences are indicated by $^{**}P \leq 0.01$ ($t = 3.805$, d.f. = 15).

($P = 0.0017$, $t = 3.805$, d.f. = 15) than parental females that transmitted '*Ca.* Phytoplasma mali' (Fig. 5). Transmitting parental females contained, on average, 13 759 $(rpl22 \times 10^4)/wg$ gene copies, while non-transmitting insects contained only 5 $(rpl22 \times 10^4)/wg$ gene copies. There was no difference detectable between '*Ca.* Phytoplasma mali' titres of parental females and males (Fig. 6).

### Potential phytoplasma acquisition on the bite spot of parental individuals

'*Candidatus* Phytoplasma mali' was detected, at a very low concentration (4.7 $(rpl22 \times 10^4)/wg$ copies), in only one individual (fourth instar nymph) derived from

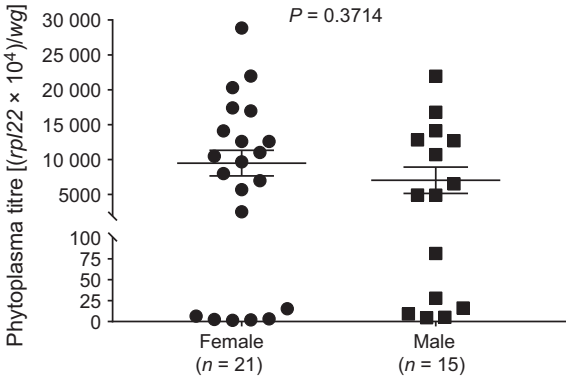


Figure 6 The titre of '*Candidatus* Phytoplasma mali' in infected *Cacopsylla picta* remigrant males and females does not differ. The phytoplasma titre, shown as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific), of individual remigrant *C. picta* females and males is represented by circles and squares, respectively. Horizontal bars show the mean, while vertical bars show the standard error of the mean for each experimental group. No significant difference between the two groups could be found ($P = 0.371 > 0.05$, $t = 0.9058$, d.f. = 34).

control group 1 (uninfected female and infected male). All other 621 tested individuals tested negative for the phytoplasma.

'*Candidatus* Phytoplasma mali' was not detected in any of the 995 tested $F_1$ individuals deriving from uninfected *C. picta* females caged with uninfected male remigrants (control group 2).

### '*Candidatus* Phytoplasma mali' infection does not affect oviposition rate or the number of eggs of *C. picta* produced

A total of 13 out of the 39 parental females (reared at Laimburg) tested positive, which corresponds to an infection rate of 33.3%. In total, 32 out of 39 (82.1%) parental females laid eggs in the net cages. Oviposition rate of uninfected (84.2%) and infected females (87.5%) caged as couples was nearly the same.

Parental females reared under controlled conditions (Fig. 7), on average, deposited similar numbers of eggs, independent of their infection status ($P = 0.3731$, $t = 0.9201$, d.f. = 14).

### Infection status of plant material

All grafts used for the net cages tested negative for '*Ca.* Phytoplasma mali'.

## Discussion

The aim of this study was to test whether '*Ca.* Phytoplasma mali'-infected *C. picta* are able to transmit the bacterium to their progeny. To address the principal question of this study, naturally infected *C. picta* individuals were collected and tested for vertical transmission of

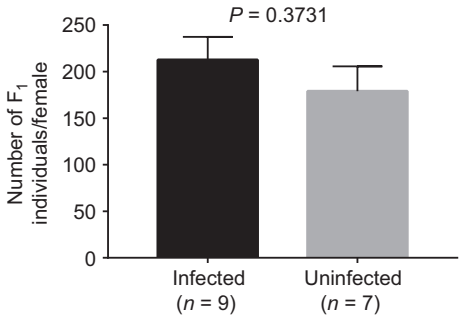


Figure 7 *Cacopsylla picta* females, infected or uninfected with '*Candidatus* Phytoplasma mali', produce similar numbers of offspring. The number of $F_1$ individuals generated by a single *C. picta* parental female (*n*) was analysed with respect to its infectious state. The black bar represents the number of $F_1$ individuals derived from infected individuals and the grey bar from uninfected individuals. Each bar shows the mean with the respective standard error of the mean. No significant differences between the two groups could be found ($P = 0.3731 > 0.05$, $t = 0.9201$, d.f. = 14).

'*Ca*. Phytoplasma mali'. As '*Ca*. Phytoplasma mali' was detected in 30.2% (average per parental female) of the egg batches and 99.1% of the $F_1$ adults, a vertical transmission of the bacteria was demonstrated. Interestingly, both the percentage of infected individuals and the phytoplasma titre gradually increased during the developmental stages, as was hypothesized by Tedeschi *et al.* (2006). Thus, it is reasonable to suggest that all eggs and early instars were actually infected, but contained very low phytoplasma concentrations, below the detection limit of the applied detection system.

The results of this study show that transmission ability is dependent on the phytoplasma load of the respective parental female, indicating that there is a critical threshold of phytoplasma concentration for vertical transmission to occur. However, it remains unclear how phytoplasma distribution in parental females can influence the transmission ability. It is possible that '*Ca*. Phytoplasma mali' must be present in the ovaries of *C. picta* to be vertically transmitted. However, further research is required to determine whether the bacteria actively migrate to the reproductive organs or are distributed all over the insect's body, and are therefore also present in the insect ovaries. Nevertheless, the infectious state of the parental female did not influence the average number of eggs produced.

The possibility that nymphs acquire phytoplasma from the bite spot on the leaf where the parental individual was initially sucking has been discussed by Arismendi *et al.* (2015). This possibility cannot be excluded by the present study as one infected nymph was found that derived from an uninfected parental female caged with an infected male. However, it can be assumed that this method of phytoplasma acquisition is quantitatively negligible in comparison to the demonstrated efficiency of transovarial transmission.

In South Tyrol, the first dramatic outbreak of AP coincided with the appearance of *C. picta*, which was previously absent in the region (Waldner, 2006; Baric *et al.*, 2011). Different studies conducted in Trentino and South Tyrol show that *C. picta* is more efficient than *C. melanoneura* in transmitting the disease and that *C. picta* populations, on average, contain a much higher percentage of infected individuals than *C. melanoneura* (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2007, 2011; Mattedi *et al.*, 2008; Baric *et al.*, 2010; Mittelberger *et al.*, 2016). Interestingly, a correlation between certain '*Ca*. Phytoplasma mali' strains and *C. picta* or *C. melanoneura* has been observed (Baric *et al.*, 2011), but no biological explanation for this correlation has been described so far. Strain differences might be responsible for a differential spread in insects and subsequent transovarial transmission, e.g. caused by bacterial adhesion and distribution properties, as hypothesized by Arismendi *et al.* (2015). Thus, the observed ability of *C. picta* to transmit the pathogen to its progeny could explain why *C. picta* is a more efficient vector of '*Ca*. Phytoplasma mali' than *C. melanoneura*.

In the present study, the phytoplasma titre in newly emerged $F_1$ adults was similar to that in infected parental individuals. Thus, it can be assumed that transovarially infected $F_1$ adults are as infective as remigrants (Mattedi *et al.*, 2008; Jarausch *et al.*, 2011). However, transmission trials with transovarially infected *C. picta* would be necessary to determine their transmission efficiency.

This study has shown that infected remigrants of *C. picta* are able to transmit the phytoplasma directly to their progeny. These findings are very important for orchard management, because they emphasize the necessity of reducing remigrant and emigrant individuals to avoid oviposition of infected eggs and the fast, exponential reproduction and spread of highly infectious insect vectors.

To the authors' knowledge, the results of this study are the first that clearly show vertical transmission of '*Ca*. Phytoplasma mali' in its insect vector *C. picta* and thus pave the way for further elucidating the molecular processes of transovarial transmission of '*Ca*. Phytoplasma mali' in *C. picta*.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Table S1.** Checklist of MIQE guidelines with details on qPCR performance of *wingless* gene quantification assay.