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Abstract. Uncovering the predation habits of small arthropods such as mites can be a major challenge, as direct observations are not always feasible in natura. Therefore, PCR-based analyses of gut content, or body content when gut dissection is not applicable, are being increasingly used to detect remains of prey items. Here, we present an integrative approach to investigate predation of eggs of the viburnum leaf beetle *Pyrrhalta viburni*, an invasive pest in North America, by the oribatid mite species *Trichoribates trimaculatus*, to assess the potential of this mite as a biological control agent. We combined (1) manipulative laboratory experiments exposing beetle eggs to mites under controlled conditions and (2) body content molecular analyses using quantitative PCR (qPCR) to detect traces of consumed *P. viburni* egg DNA in mites. To account for risks of false-negative and false-positive results, mainly due to detectability problems, body surface contamination, and non-specific DNA amplification, we developed a stepwise procedure to analyze qPCR results. Egg integrity and survivorship were unaffected by mites in all experiments. However, traces of *P. viburni* DNA were detected in the body of mites exposed to *P. viburni* eggs, suggesting that they consumed either fragments of the chorion or fragments of the egg cap secreted by *P. viburni* females to protect the eggs, which was shown to contain *P. viburni* DNA. In conclusion, *T. trimaculatus* does not directly impact *P. viburni* eggs and should therefore not be considered as a biocontrol agent. The fact that mites did not directly predate eggs but contained traces of *P. viburni* DNA shows that detection of an organism's DNA within a presumed predator does not necessarily equal predation and that results of molecular analyses should be interpreted with caution in studies attempting to reconstruct trophic interactions.

Key words: biological control; gut content analysis; insect-mite interactions; oribatida; predation; qPCR; trophic networks; viburnum leaf beetle.

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Introduction

Understanding predation and complex trophic interactions in natura is critical for community ecologists and for scientists working in the fields of pest management and biological control (Gariepy et al. 2007, Eitzinger et al. 2019). The feeding habits of minute or inconspicuous predatory species can rarely be directly observed in the field, making the identification of what they eat under natural conditions often fragmentary. As a result, trophic networks involving minute organisms are often under-explored and key trophic interactions may go unnoticed. In the last two decades,

significant advances in molecular detection of prey DNA in the digestive system, guts, regurgitates, or whole body of predators have revolutionized community ecology studies and have been applied in a wide range of terrestrial and aquatic systems (Sheppard and Harwood 2005, King et al. 2008). Conventional PCR methods have been extensively applied to determine the presence of prey DNA within the bodies of putative predators, to measure the frequency of predation events, and to assess the impact of predators on prey populations (Symondson 2002, Cuthbertson et al. 2003, Gariepy et al. 2007, 2018, Gagnon et al. 2011, Rivera-Rivera et al. 2012). In contrast, the quantitative polymerase chain reaction (qPCR) has only been recently adapted for investigations of predator–prey interactions (Lundgren et al. 2009, 2014, Weber and Lundgren 2009, Prischmann-Voldseth and Lundgren 2011, Huang et al. 2016) and, to our knowledge, only few studies based on qPCR have been applied to mites (Prischmann-Voldseth and Lundgren 2011, Pérez-Sayas et al. 2015). The qPCR approach has significant advantages over conventional PCR due to its sensitivity, speed, and robustness in addition to the information provided by relative quantification and melting curve analysis of amplification products (Bastien et al. 2008). However, its high performance could be seen as a double-edged sword, because it requires performing preliminary laboratory qPCR assays to optimize the method and to assess the occurrence of prey consumption with reliability (Weber and Lundgren 2009, Prischmann-Voldseth and Lundgren 2011, Pérez-Sayas et al. 2015).

Here, we developed an integrative approach combining molecular analyses and manipulative laboratory experiments to investigate predation of eggs of the viburnum leaf beetle *Pyrrhalta viburni* (Paykull) (Coleoptera: Chrysomelidae) by the oribatid mite *Trichoribates trimaculatus* (Koch) (Oribatida: Ceratozetidae). *Pyrrhalta viburni* is native to Eurasia and is an invasive pest of *Viburnum* trees and shrubs in North America. This univoltine species overwinters as eggs and feeds on leaves of *Viburnum* shrubs in the larval and adult stages, often killing shrubs after 2–4 years of repeated complete defoliations (Weston and Desurmont 2002, Weston et al. 2007). Finding biological control agents that could be used either through augmentative methods (Desurmont and Weston 2008*a*, *b*) or through classical biological control (Desurmont 2009) is a critical issue for *P. viburni* management. High densities of the oribatid mite *T. trimaculatus* were recently found in egg masses of *P. viburni* in its native range (Fig. 1), specifically in southern France (Desurmont et al. 2019). This mite species has a wide distribution in the holarctic area and is known to occur in mosses and on the bark of diverse shrubs or trees (Kehl and Weigmann 1992, Smrž 2006). Previous behavioral and histological observations revealed that it can feed on algae and plant fragments but avoids certain fungi (Smrž 2006), but its presence in *P. viburni* egg masses had never been reported prior to 2019 and its ecology and dietary habits within this specific environment are unknown. Egg masses are laid in cavities dug by *P. viburni* females in young Viburnum twigs, and the eggs are covered with a protective secretion produced by females (egg cap). It is possible that this particular mite species inhabits *P. viburni* egg masses because (1) egg masses provide shelter and/or oviposition sites for the mite, and (2) *P. viburni* egg masses constitute a food source for the mite. Mites may potentially feed on the eggs themselves, the protective egg cap, micro-organisms associated with *P. viburni* egg masses, or wood fragments. The present study aimed at answering the main question: Does *T. trimaculatus* directly consume *P. viburni* eggs and significantly impact egg survivorship? Answering this question would help estimating the potential of *T. trimaculatus* as a biological control agent. The

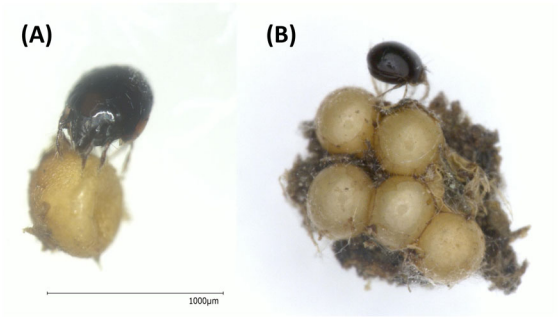

Fig. 1. Pictures of a *Trichoribates trimaculatus* adult on (A) a single *Pyrrhalta viburni* egg and on (B) a *P. viburni* egg mass. The brown matter visible in the background of the eggs in (B) is the protective secretion produced by *P. viburni* females that covers the eggs in nature.

use of mites as insect egg predators has been use of mites in biological control of insects has a long and rich history (McMurtry and Croft 1997, Gerson et al. 2003). For example, eggs and larvae of the western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) are preyed upon by several generalist-feeding soil-dwelling Astigmata and Mesostigmata mites (Brust and House 1988, Prischmann et al. 2011). In addition to the main experimental question, this study was also an opportunity to measure fungal and algal growth on *P. viburni* egg masses in the presence or absence of mites in order to gain additional insights on their feeding habits and interactions with micro-organisms in this specific environment.

An integrative approach has been developed to answer the main experimental question. First, manipulative laboratory experiments were conducted to determine the impact of *T. trimaculatus* mites on *P. viburni* egg under different controlled conditions. Second, molecular qPCR protocols were developed to detect *P. viburni* DNA traces within mite bodies. In order to overcome issues associated with the qPCR method (i.e., detectability problems, DNA contamination on body surface, and non-specific DNA amplification; King et al. 2008, Weber and Lundgren 2009, Remén et al. 2010, Prischmann-Voldseth and Lundgren 2011), a stepwise procedure was elaborated to validate the results of the molecular analyses. This stepwise procedure is not limited to mite predators or even to predator–prey interactions and could theoretically be used for any investigation of trophic interactions using the qPCR method. The integrative approach presented here has multiple benefits: Manipulative experiments under controlled conditions allow for a direct quantification of the impact of mites on *P. viburni* eggs, and qPCR analyses provide a proof of consumption of egg DNA by mites. Together, these results should be sufficient to assess the potential of *T. trimaculatus* as a possible biological control agent against *P. viburni*.

Materials and Methods

Species studied

Pyrrhalta viburni eggs are laid in groups of 6–12 eggs in small cavities dug by females in the pith of young twigs of *Viburnum* shrubs. These cavities are covered with a protective frass-like secretion capsule produced by females (egg cap), and such egg masses are typically found clustered on the underside of *Viburnum* twigs (Weston et al. 2008, Desurmont and Weston 2011). For all experiments, *P. viburni* egg masses were collected in natura on *Viburnum tinus* L. shrubs naturally infested by *P. viburni* (Montpellier area, France), and *T. trimaculatus* adults were collected on *V. tinus* shrubs present in the same area during the days preceding each experiment (Fig. 1). Mite identity was assessed through morphological examination, based on previous observations in the same geographical area (Desurmont et al. 2019). Voucher specimens of *T. trimaculatus* have been deposited in the acarological collection of the Institute of Biology, University of Graz (curated by T. Pfingstl and J. Baumann). Once collected, mites were kept in plastic boxes on *V. tinus* twigs without *P. viburni* egg masses at ambient temperature and photoperiod (natural sunlight) for at least 3 d before being used for experimental purposes.

Predation experiments

Three successive experiments were designed to study predation of *P. viburni* eggs by *T. trimaculatus* with slightly different conditions of conservation of *P. viburni* eggs set to increase their survivorship (the percentage of intact eggs in the control treatment increased from 38.3% to 95.1% between experiment 1 and experiment 3; Appendix S1: Tables S1, S3, S4, and S5). In the three experiments, *P. viburni* eggs were exposed to *T. trimaculatus* mites in petri dishes, and their integrity was compared to control eggs at the end of the exposure period. The integrity of *P. viburni* eggs was visually evaluated based on three categories: intact, damaged, and pierced. Intact eggs were round and turgescent and did not show any sign of damage. Damaged eggs were not turgescent and showed early or advanced shriveling but were not pierced. Pierced eggs were eggs whose chorion was damaged to the point of rupture. Our assumption is that pierced eggs are more likely to have been preyed upon than damaged eggs. In addition to egg integrity, the number of live and dead mites was also counted at the end of the experiments, and *P. viburni* larval emergence was recorded to calculate egg survivorship in experiment 3 (beetle eggs did not

hatch in experiments 1 and 2). The three experiments were conducted in a growth chamber kept at a 12/12 (l/d) photoperiod. The temperature of the growth chamber was 25°C for the first and second predation experiments, and was 20°C for the third experiment.

The modalities of each experiment were as follows. In the first experiment, sections of *Viburnum* twigs infested with 1–4 egg masses were placed in 20 individual tubes (3.5 × 7 cm diameter) closed by a screwed cap with a thin mesh (500 μm diameter), and a wet piece of dental roll (Coltène/Whaledent, Cuyahoga Falls, Ohio, USA) was placed inside each tube to provide moisture. Twenty *T. trimaculatus* adults were added per tube for half of the tubes (mites treatment), and the other half remained free of mites (control treatment), for a total of ten replicates per treatment. The exposure period was one month (12 Sept–13 Oct 2017), and egg masses were opened at the end of the exposure period to count the eggs present inside and evaluate their integrity. Because eggs did not hatch at the end of this experiment, possibly due to desiccation, the second predation experiment was designed to increase the moisture level in the petri dishes and to shorten exposure time.

For the second experiment, four *P. viburni* eggs were taken from egg masses and directly placed in 20 petri dishes (5.5 cm diameter; Falcon plastics, California, USA) on a thin layer of 1.5% water agar (Sigma Chemical, St. Louis, Missouri, USA). Ten *T. trimaculatus* adults per petri dish were added for half of the petri dishes (mite treatment), and the other half of the petri dishes remained free of mites (control treatment), for a total of ten replicates per treatment. All petri dishes were then sealed with Parafilm (American National Can, Chicago, Illinois, USA). The exposure period was 14 d (04 October–18 October 2017). Increased moisture allowed fungi to proliferate within the petri dishes. Pictures of the petri dishes were taken at the end of the experiment, and the surface of the petri dish covered by fungal growth was measured (cm^2) using the software ImageJ for Windows version 1.48v (Schneider et al. 2012). Eggs from the second experiment did not hatch, despite the increased moisture. We hypothesized that direct contact between eggs and water agar formed a thin pellicle of water around the eggs, eventually killing them. The third predation experiment was thus designed to balance the high humidity necessary to prevent egg desiccation and the need to avoid direct contact between eggs and water agar.

For the third experiment, 3–6 *P. viburni* eggs were taken from egg masses and directly placed in 40 petri dishes (5.5 cm diameter) with a thin layer of 1.5% water agar (Sigma Chemical) that was covered by two Whatman glass microfiber filters (GE Healthcare, Chicago, Illinois, USA). Ten *T. trimaculatus* adults per petri dish were added to half of the petri dishes (mite treatment), and the other half of the petri dishes remained free of mites (control treatment), for a total of 20 replicates per treatment. All petri dishes were then sealed with Parafilm. The exposure period was 14 d (22 December 2017–5 January 2018). After examination of the integrity of the eggs and counting live and dead mites, all mites were removed and the petri dishes were sealed again with Parafilm for egg hatch monitoring. The number of larvae that hatched from each petri dish was recorded. The Whatman glass microfiber filters drastically limited fungal growth but did not prevent algal growth. Pictures of the petri dishes were taken at the end of the experiment, once all larvae had hatched (21 March 2018). The surface covered by algae was measured (cm^2) for all petri dishes using the software ImageJ for Windows version 1.48v (Schneider et al. 2012).

The mites originating from predation experiment 3 were immediately frozen by transferring them alive to tubes placed in a freezer at −24°C at the end of the experiment and kept at −24°C subsequently, and a subset of these mites was used for DNA extractions. This subset consisted of four mites originating from 13 replicates of the experiment (52 mites total). Additionally, if a mite from one replicate was found to contain *P. viburni* DNA, all the remaining mites from this replicate were also used for DNA extractions (17 additional mites). All DNAs were extracted and analyzed by qPCR for predation detection following the qPCR procedure described below.

Development of the qPCR procedure for detecting predation

Designing a reliable procedure to detect *P. viburni* DNA within the body of small mites was a multi-step process.

Genomic DNA extraction.—We started with the optimization of a genomic DNA extraction workflow using locally collected (1) *T. trimaculatus* adults that had been starved for at least 3 d in petri dishes to insure they did not contain *P. viburni* DNA and (2) four clumps of eggs (11, 20, 30, and 50 eggs) of *P. viburni* to characterize prey DNA signal. Once this step optimized, DNA extraction was carried out for all mites from the third predation experiment and from the time-lapse trials (described below). The complete protocol is available in Appendix S1. Briefly, under the optimized workflow, each specimen prior extraction was bathed in molecular grade water at 56°C three times. The third rinse water was always kept for qPCR detection (procedure described *Body surface contamination (risk of false positives type 1)*). Genomic DNA extractions were performed using DNeasy Blood and Tissue extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Choice of the targets and design of qPCR primer sets.—As it is generally admitted that the qPCR performance depends on a large number of methodological factors among which (1) the choice of the target sequence and (2) the design of the primers which dictate the specificity and the sensitivity of the detection by qPCR, these factors were prioritized for developing in silico the qPCR procedure presented in this study.

The markers targeted were the barcode region in the Cytochrome Oxidase Subunit I (*CO1*) and the 5′ region of the nuclear SSU rDNA (18S) as both fulfill the criterion of a high copy number per cell which maximizes the sensitivity of predation detection (Pérez-Sayas et al. 2015). The complete procedure for the design of qPCR primer sets is available in Appendix S1. Briefly, *COI* and 18S were amplified in *P. viburni* eggs and starving mites. *CO1* and 18S sequences obtained in this study were merged with respective homologous sequences retrieved from Genbank database and listed in Appendix S1: Table S2. *P. viburni* specific qPCR primers were designed from the *CO1* and 18S alignments using the online software Primer 3. The size of the targeted *CO1* fragment in *P. viburni* egg DNA was 116 bp, and primer sequences were as follows: Pyr-*CO1*-F (5′-TGAGCTGGAATAGTAGGAAC-3′) and Pyr-*CO1*-R (5′-AAAGCATGAGCAGTAACAAT-3′) (Appendix S1: Fig. S2). The size of the targeted 18S fragment in *P. viburni* egg DNA was 214 bp, and primer sequences were as follows: Pyr-18S-3F (5′-ACCCACATTTACTTGGATAA-3′) and Pyr-18S-3R (5′-ACAGTTGATAAGGCAGACAT-3′).

Examination of applicability of the markers for detecting potential predation.—The complete procedure for testing the applicability of the markers to predation detection is available in Appendix S1. Briefly, specificity of the primers (ability of a primer set to amplify a unique product at low initial template concentration, i.e., prey egg DNA alone [1–100 pg/μL] or prey egg DNA spiked into 100 pg/μL DNA of starving mite) was tested first by analyzing the dissociation plot and confirmation of the size of the amplicon using agarose gel analysis.

Optimized qPCR procedure.—For both markers, the qPCR was carried out in a 20 μL total volume composed of 10 μL 2 × Sso Advanced SYBR Green master mix (Biorad, Hercules, California, USA), 250 nmoles/L of each specific qPCR primer, 0.4 mg/mL Bovine Serum Albumine (BSA), 5.4 μL pure water, and 2 μL of DNA template. The qPCRs were run in a CFX96 system (Biorad) under the following conditions: 95°C for 3 min, followed by 45 cycles of 95°C for 5 s, 54°C for 10 s, and 72°C for 30 s. A dissociation or melt curve was created for each reaction. After PCR cycling, samples were heated to 95°C for 10 s and cooled to 65°C for 15 s at a linear transition rate of 0.3°C/s and fluorescence monitored continuously. The CFX96 software calculates the melting temperature (Tm) for each positive reaction which corresponds to 76.8°C and 85.2°C for the *CO1* and 18S amplicons of *P. viburni* DNA, respectively (and confirmed by sequencing). We considered that reactions that did not amplify within 45 cycles were non-detected. On each 96-well plate, along with the unknown samples, we added a negative DNA control (mites that had been starved), negative PCR control (no template), and a positive control series, i.e., *P. viburni* egg DNA (100–0.01 pg/μL) alone or spiked control into 100 pg/μL of mite DNA. This spike control reaction assessed whether the mite samples contain any components that would inhibit the PCR, resulting in false negative (see *Materials and Methods: Detectability problems (risk of false negative)*). DNA extracts from predators can be of poor quality, yielding false-negative results and dramatically lowering prey detection rate (Juen

and Traugott 2006). Each positive sample generated a C_t (the threshold PCR cycle where fluorescence was distinguishable from background), which has an inverse relationship with the initial quantity of *P. viburni* egg DNA. For each mite cohort assayed during the experiments, the proportion of mites tested positive for *P. viburni* DNA, as well as the mean quantity of prey DNA (represented as $Ct^{-1} \times 100$) was calculated. To confirm qPCR result in each mite tested positive, amplicon was sequenced following the procedure described above.

Rate of decay in detectability: time-lapse trials.—The most common technical issue affecting assay performance lies in the fact that the probability of detecting prey post ingestion decreases with time, as prey DNA is degraded over the course of digestion (Weber and Lundgren 2009, Huang et al. 2016). In a study of the dynamic interaction between the *Coleomegilla maculata* predator and its Colorado potato beetle prey (*Leptinotarsa decemlineata*), Weber and Lundgren (2009) evidenced a severe reduction in detectable DNA within minutes post ingestion. Furthermore, when gut dissection is not applicable, and DNA is extracted from whole predators, successful amplification of prey DNA is likely to be negatively affected by the low prey/predator DNA ratio, especially degraded prey DNA (King et al. 2008). Therefore, in order to estimate the rate of decay in detectability, we conducted time-lapse trials designed to evaluate how long *P. viburni* DNA remained detectable within the mite during the digestion process. We used mites that had been starved beforehand and then exposed to a fresh *P. viburni* egg puree (i.e., eggs smashed and smeared in a petri dish) during three hours. Eggs were exposed as a puree to maximize the probability that mites would consume some of the egg cells. After the exposure period, mites were maintained without food during 0, 6, 12, 24, and 48 h. Ten mites per time point were tested. Mites were frozen immediately after the desired post-exposure period at −24°C. With the 18S-qPCR primer sets, the results of this trial were not conclusive as no amplification was obtained in all samples although confirmed positive with *CO1* primer set, and no clear dissociation plots but obvious random dsDNA formation. Therefore, the 18S-qPCR primer set was not retained for the predation trial.

Pitfalls of the qPCR analysis and development of a stepwise procedure to process qPCR results

We faced some inconsistent results over the course of preliminary applications of qPCR on *T. trimaculatus* mites, including false-negative and false-positive results. Specifically, the pitfalls we encountered and the solutions we adopted to overcome them are described in the following paragraphs. These optimization efforts of our qPCR methodology led to the development of a stepwise procedure for processing qPCR results, which is summarized in Table 1.

Detectability problems (risk of false negatives).—To account for the presence of possible PCR amplification inhibitors in our samples, we diluted the template DNA of a sub-sample of the mites assayed that tested negative, and retested them. We also added spiked control, which consisted in the addition of a determined quantity of *P. viburni* DNA, to these samples, thereby ensuring that amplification of prey DNA was effectively successful. We systematically added in the qPCR buffer a PCR facilitator such as BSA, which was found to improve amplification of DNA from soil-dwelling predators by Juen and Traugott (2006).

Body surface contamination (risk of false positives type 1).—Surface contamination by prey DNA needs to be considered when developing a qPCR assay for detecting predation without dissecting the gut, especially when arthropods regurgitate gut contents (Remén et al. 2010, Prischmann-Voldseth and Lundgren 2011). The main risk is to amplify prey DNA passively present on the cuticle of the predator but not ingested, leading to false-positive results. During the course of the preliminary application of the qPCR procedure to mites, we evidenced that the later rinse water in which the mites were bathed prior to extraction could contain prey cells and DNA subsequently detected by qPCR, even after three extensive rinsings. We hypothesized that some prey cells could still be entrapped on the cuticle or in the chelicerae of the mites, potentially introducing a bias to the results. Therefore, for all predation and feeding assays, we always kept the later rinse water for any mite assayed and tested it for the presence of prey DNA. We only

Table 1. Summary of the methodology developed for the molecular analyses of *Trichoribates trimaculatus* body content and main results obtained.

Experimental question	Methodology used	Main result
1. Rate of decay in detectability: time-lapse trials How long does *P. viburni* DNA remain detectable after ingestion by *T. trimaculatus* mites?	Time-lapse trials using 5 time points (0, 6, 12, 24, 48 h) post-exposure to *P. viburni* eggs	*P. viburni* remained detectable up to 12 h after ingestion by *T. trimaculatus* (Table 2)
2. Detectability problems (risk of false negatives) How to account for the risk of not amplifying *P. viburni* DNA effectively ingested by *T. trimaculatus*?	Dilution of the extracted DNA to overcome PCR inhibitors, and addition of spike control, which consisted in the addition of a determined quantity of *P. viburni* DNA	Out of the 69 mites tested, 30 that tested negative were chosen and went through this procedure. No additional positive samples were found
3. Body surface contamination (risk of false positives type 1) How to account for the risk of detecting *P. viburni* DNA passively carried by *T. trimaculatus*?	Every mite was rinsed three times prior to extraction. If the mite was positive after the qPCR, the third rinse water was tested for presence of *P. viburni* DNA. If the rinse water tested positive, the sample was discarded from the analysis	Out of the 69 mites tested, 39 mites tested positive in at least one of the three qPCRs that were run independently. Out of these 39 mites, 4 were discarded because rinsing baths tested positive, and 35 were retained
4. *Amplification of non-prey DNA (risk of false positives type 2)* How to account for the risk of amplifying DNA not belonging to *P. viburni* through qPCR?	Every mite that tested positive once was retested in two other qPCR runs. Mites for which Tm of the amplicon was different from 76.8 and not repeatedly positive over the runs were discarded from the analysis	Out of the 35 mites that tested positive, 5 were retained as they were tested positive in at least two separate qPCRs runs with a Tm of the amplicon of 76.8 (confirmed by sequencing)
5. Does *T. trimaculatus* consume *P. viburni* egg DNA?	Molecular analysis by qPCR of the body content of 69 *T. trimaculatus* mites exposed to *P. viburni* eggs	Out of 69 mites assayed, 5 were considered true positives for containing *P. viburni* DNA

Note: Although question 5 (Does *T. trimaculatus* consume *P. viburni* egg DNA?) was the main experimental question, questions 1, 2, 3, and 4, had to be answered to interpret the results correctly.

considered as a positive signal of predation or feeding any positive qPCR amplification from mite extracted but negative from rinse water.

Amplification of non-prey DNA (risk of false positives type 2).—The high sensitivity of the qPCR may lead to amplification of non-prey DNA. To account for this risk, all mite samples that tested positive for *P. viburni* DNA once were retested in two other qPCR runs. Mites that did not repeatedly test positive over the runs were considered false positives and discarded from the analyses.

Statistical analyses

Differences in the counts of intact, damaged, and pierced eggs (predation experiments 1, 2, and 3) as well as differences in counts of viable and non-viable eggs (predation experiment 3) between the mites treatment and the control treatment were compared using Fisher's exact test ($\alpha = 0.05$). For experiment 1, replicates containing an abnormally high number of eggs (>10) were excluded from the analyses (3 replicates out of 20). Differences in surfaces covered by fungal growth (predation experiment 2) and algal growth (predation experiment 3) between the mites and the control treatments were compared using non-parametric Wilcoxon sum of ranks tests ($\alpha = 0.05$). Differences in the quantity of prey detected after feeding ($Ct^{-1} \times 100$) between the different time points were compared using a non-parametric Kruskal-Wallis test ($\alpha = 0.05$). The software used to perform the statistical analyses was JMP Pro Version 12 (SAS Institute, Cary, North Carolina, USA).

Results

Predation experiments

There was no significant effect of mites on the integrity and survivorship of *P. viburni* eggs in any of the three predation experiments. At the end the first experiment, the proportions of intact eggs and eggs damaged or pierced did not differ between control eggs and eggs exposed to mites (Fisher's exact test, $P = 0.10$, Fig. 2A). There was in average 8.3 ± 0.9 mites alive per tube for the mite treatment, representing $42.8 \pm 4.3\%$ mite survivorship. At the end of the second experiment, there was again no difference in the

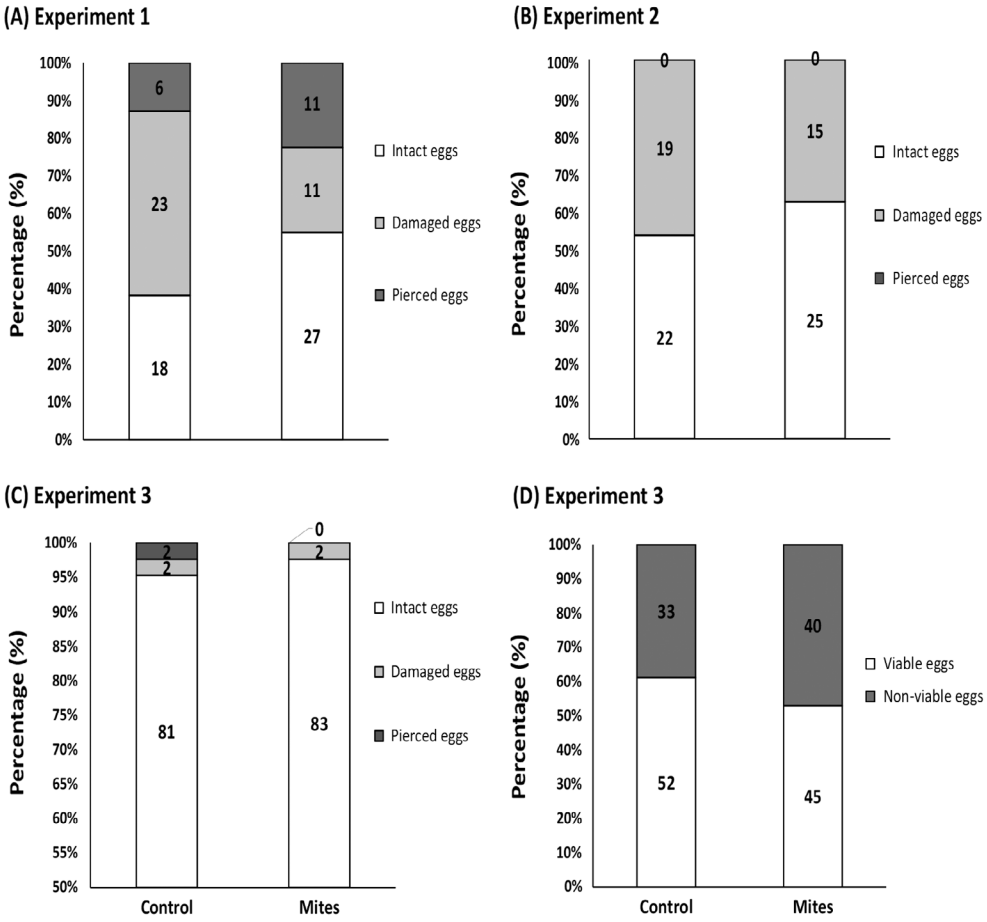

Fig. 2. *Trichoribates trimaculatus* does not negatively affect *Pyrrhalta viburni* eggs. (A–C) Overall percentage of intact, damaged and pierced *P. viburni* eggs after exposure to *T. trimaculatus* (Mites) compared to unexposed eggs (Control) in three different experiments. (D) Percentage of viable (emergence of a larva) and non-viable (no larval emergence) eggs from experiment 3. Numbers within each bar represent the total number of eggs belonging to each treatment (number of replicates per treatment per experiment: experiment 1, $N = 10$; experiment 2, $N = 10$; experiment 3, $N = 20$). There were no significant differences among the relative proportions of the different categories of eggs (intact, damaged, and pierced, or viable and non-viable) between the two treatments (Control and Mites) for any of the experiments (Fisher's exact test, $\alpha = 0.05$).

proportions of intact and damaged eggs between control eggs and eggs exposed to mites ($P = 0.55$, Fig. 2B). No pierced eggs were observed during this experiment. There was in average 6.9 ± 0.7 mites alive per petri dish for the mite treatment at the end of the exposure period, representing 75.8 ± 4.7% mite survivorship. In this experiment, fungal growth was 2.7 times higher in control petri dishes than in petri dishes exposed to mites (control 1.36 ± 0.19, mites 0.50 ± 0.09; $\chi^2 = 11.6$, $P < 0.0001$; cm^2, mean ± SE; Fig. 3A). Finally, at the end of the third experiment, the proportions of intact, damaged, and pierced eggs still did not differ between control eggs and eggs exposed to mites ($P = 0.55$, Fig. 2C). There was in average 8.3 ± 0.9 mites alive per petri dish for the mite treatment at the end of the exposure period, representing a 42.8 ± 4.3% mite survivorship. The proportions of viable and non-viable eggs did not differ between control eggs and eggs exposed to mites ($P = 0.35$, Fig. 2D), although it should be noted that there were three replicates with 0% egg survivorship in the mite treatment, and none

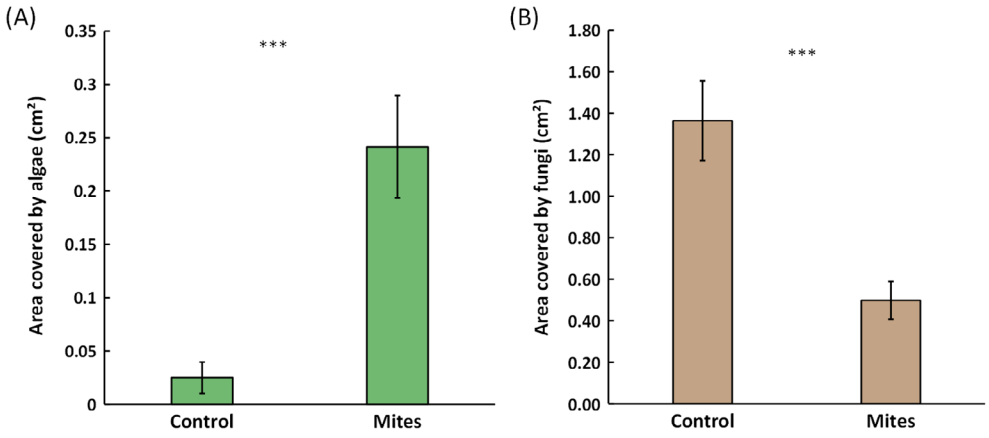

Fig. 3. Associations between mite presence and growth of fungi and algae. (A) Area covered by fungi (cm^2, mean ± SE) in petri dishes containing *Pyrrhalta viburni* eggs after exposure to *Trichoribates trimaculatus* (Mites) compared to petri dishes with unexposed eggs (Control). The difference between treatments is statistically significant ($P < 0.0001$, Wilcoxon sum of ranks test, $\alpha = 0.05$). (B) Area covered by algae (cm^2, mean ± SE) in petri dishes containing *P. viburni* eggs after exposure to *T. trimaculatus* (Mites) compared to petri dishes with unexposed eggs (Control). Asterisks indicate a significant difference between treatments ($P < 0.0001$, Wilcoxon sum of ranks test, $\alpha = 0.05$).

in the control treatment (the minimum egg survivorship was 25% for the control; Appendix S1: Table S5). Algal growth was 10 times higher in petri dishes with eggs exposed to mites than in petri dishes with control eggs (control 0.02 ± 0.01, mites 0.24 ± 0.05; $\chi^2 = 18.5$, $P < 0.0001$; cm^2, mean ± SE; Fig. 3B).

Molecular analyses

Rate of decay in detectability: time-lapse trials.—*Pyrrhalta viburni* egg DNA was detected with the *CO1*-qPCR primer set repeatedly (based on three separate qPCR runs and three replicates per run) in nine mites that had been exposed to *P. viburni* egg puree, and in particular in four mites that were frozen immediately after exposure (time point 0 h). Detection was still possible 6 and 12 h after exposure, albeit less frequently (Table 2). All positive reactions were confirmed by sequencing. Three mites that tested negative at first were retested in order to discard false negative (Table 1) and were found positive after the second test.

Detection of P. viburni *egg DNA in mites from predation experiment 3.*—In predation experiment 3, three mites were tested positive for *P. viburni* egg DNA out of 52 mites sampled in 13 replicates. These three mites originated from three different replicates (#9, #15, and #17). The DNA of all the remaining mites from these replicates (17 mites in total) was also extracted, and two additional positive mites were observed (both originating from replicate #9; Table 1). The quantity of *P. viburni* egg DNA detected was 2.55 ± 0.07 ($Ct^{-1} \times 100$; mean ± SD) in replicate 9, 2.60 ± 0.00 ($Ct^{-1} \times 100$; mean ± SD) in replicate #15, and 2.77 ± 0.00 ($Ct^{-1} \times 100$; mean ± SD) in replicate #17. All eggs from replicates #15 (three eggs) and #17 (four eggs) were intact at the end of the experiment, and replicate #9 had one damaged egg (not pierced) and two

Table 2. Percentage of successful *Pyrrhalta viburni* DNA detection for mites exposed to *P. viburni* egg puree and in case of positive detection, the quantity of prey DNA ($Ct^{-1} \times 100$) corresponding C_t values (na = non-applicable).

Time (h)	Percent positive (*N*)	*P. viburni* egg DNA
0	40 (4/10)	2.70 ± 0.12
6	20 (2/10)	2.57 ± 0.04
12	33 (3/9)	2.56 ± 0.03
24	0 (0/10)	na
48	0 (0/10)	na

Note: Time is post-exposure to *P. viburni* egg puree; egg DNA ($C_t - 1 \times 100$; mean ± SD).

intact eggs. Percentage larval emergence was 66.7, 0.0, and 50.0 in replicates #9, #15, and #17, respectively. The fact that positive samples were obtained from replicates where all eggs remained intact shows that the DNA of *P. viburni* detected in the mites did not always originate from predated eggs. One alternative possibility is that *P. viburni* DNA is present in the protective secretion covering the eggs and that this secretion was consumed by the mites. Thus, to further explore this possibility, we extracted genomic DNA from three batches of *P. viburni* protective secretion capsules collected on 4 September 2018. A 1/100 dilution of the DNA extracted (three replicates per batch) was amplified by qPCR as described in the Materials and Methods section. Amplifications were all positive and corresponded to the same single amplicon (Appendix S1: Fig. S2) as confirmed after sequencing. The quantity of *P. viburni* DNA detected per batch of secretion capsule was estimated to be 2.84 ± 0.05 ($Ct^{-1} \times 100$; mean $\pm$ SD), which is above the values obtained within the mites assayed in the predation experiment 3.

Discussion

Usefulness of qPCR for detecting prey DNA within a mite body

A qPCR assay for the detection of *P. viburni* eggs consumed by *T. trimaculatus* was developed and successfully tested using the *CO1* region. This is, to our knowledge, the first fully described system of predation detection using qPCR in oribatid mites and the third one in mites (Prischmann-Voldseth and Lundgren 2011, Pérez-Sayas et al. 2015). The 18S region turned out to be inappropriate for the detection of feeding in our system contrary to *CO1*, stressing out the importance of targeting different regions. *CO1* has been the most used target region in molecular analyses of predation in invertebrates systems (Juen and Traugott 2006, Weber and Lundgren 2009), and in mites in particular (Prischmann-Voldseth and Lundgren 2011, Rivera-Rivera et al. 2012). We confirmed that the mites could effectively feed on the egg puree and that the detection times for the feeding experiment are within the range of what other similar studies with mites have found (Prischmann-Voldseth and Lundgren 2011, Rivera-Rivera et al. 2012, Pérez-Sayas et al. 2015).

During the development of the qPCR approach, we identified and addressed several pitfalls to avoid both false-positive and false-negative results, which proved critical for the interpretation of the results. Indeed, 87.1% of the samples initially considered positive were later discarded as false-positive samples (34 out of 39 samples; Table 1). Surface contaminations by prey DNA could account for false positive results. Similar results were obtained by Greenstone et al. (2012) and Remén et al. (2010). Remén et al. (2010), using the fungal feeding oribatid mite *Archegozetes longisetosus*, demonstrated that surface contamination was a serious problem and that both washing and dissection were needed to remove surface contamination on such small organisms. Greenstone et al. (2012), using a 2.5% bleach treatment during 40 min, were able to oxidize most DNA external contamination while sparing target DNA in the gut of their two predators tested, *Podisus maculiventris* and *Coleomegilla maculata* (Greenstone et al. 2012). Therefore, surface contamination by prey DNA needs to be considered more systematically when developing qPCR approach for detecting predation. We also evidenced during the time-lapse trials false-negative results mainly due to PCR inhibition, which was overcome by simply diluting the template DNA, although we took the risk that further dilution may jeopardize detection (King et al. 2008).

To remain cautious with our conclusions, and owing to the limited number of positive amplicons obtained, we sequenced these amplicons. However, we are aware that this cannot be applied on a large number of samples as it would call into questions the cost and the justification for developing a qPCR approach. Therefore, the present approach leaves much ground for future improvement to circumvent the need to sequence every result. To overcome this challenge, we would suggest to investigate the simultaneous amplification of different regions by qPCR (Multiplex qPCR) as it was intended at the beginning of the study with the choice of two markers (i.e., 18S and the *CO1*).

Predation of P. viburni *eggs by* T. trimaculatus

Our study shows unambiguously that the oribatid mite *T. trimaculatus* does not prey upon *P. viburni* eggs. Three consecutive predation experiments yielded the same results: Exposure to *T. trimaculatus* adults did not have any impact

on *P. viburni* eggs or larval emergence. The densities of mites used in these experiments were high but not unrealistic compared to natural densities observed on *P. viburni*-infested *V. tinus* shrubs in nature (Desurmont et al. 2019). However, despite the lack of direct predation observed in the experiments, traces of *P. viburni* DNA were found inside the body of several mites from the third predation experiment, indicating that these mites did in fact consume something that contained *P. viburni* DNA. It is possible that mites grazed at the surface of the egg membrane, ingesting some egg DNA in the process. It is also possible that mites fed on the protective secretion that *P. viburni* females produce to protect the eggs (egg cap), which was shown to contain *P. viburni* DNA. It is very hard to separate entirely the eggs from the secretion without damaging the eggs, and remains of this secretion were still coating the eggs during the experiments. The fact that the rinsing water that was used to clean up mite specimens before molecular extraction tested positive for *P. viburni* DNA in several occasions also shows that mites might passively carry material containing *P. viburni* DNA on their body surface, and highlights the importance of rinsing procedures for investigations of predation events using gut or body content molecular analyses.

Effects of mite presence on fungal and algal growth

Finally, although it did not affect *P. viburni* eggs, the presence of *T. trimaculatus* mites had a strong effect on fungal and algal growth. Fungal growth was strongly reduced in presence of mites in experiment 2, and algal growth was strongly enhanced after exposure to mites in experiment 3 (Fig. 3). These results suggest that *T. trimaculatus* may directly consume and/or prevent the growth of fungi associated with *P. viburni* eggs. Many oribatid mites are known for their mycophagous feeding habits (Mitchell and Parkinson 1976, Schneider and Maraun 2005, Schneider et al. 2005). However, the species *T. trimaculatus* has been shown to avoid fungi and to mainly consume algae (Smrž and Čatská 2010). This finding was based on experiments using only one group of fungi (*Stachybothris*) as food source (Smrž 2006). Therefore, it is possible that *T. trimaculatus* may feed on certain fungi but refuses others. It is also possible that mites in our experiments did not consume fungi but that their presence somehow disturbed fungal growth over the course of the experiment. Regarding algal growth, the presence of *T. trimaculatus* may promote the dispersal and growth of algae around *P. viburni* eggs through passive transportation on the cuticle and/or through dissemination after digestion (Behan and Hill 1978, Hubert et al. 2001, Hofstetter and Moser 2014). It is interesting to note that algae grew in almost every petri dish with mites present (19 out of 20 replicates) but were totally absent of half of the control petri dishes (10 out of 20 replicates). Therefore, the possibility that mites themselves may have been the source of algae in some replicates is not to be excluded. Some Oribatid mites are known to carry fungal spores on their bodies and contribute to their dispersal in forest soils (Behan and Hill 1978, Hofstetter and Moser 2014), and the same may be true for algal resting spores. To further elucidate the roles played by fungi and algae in the feeding habits of *T. trimaculatus* (Smrž and Čatská 2010), the integrative approach developed for this study could be easily applied to investigate in further experiments the presence of fungal and algal DNA inside the body of *T. trimaculatus* mites.

Conclusion and Perspectives

Our study sheds light on some of the feeding habits of *T. trimaculatus* and its interactions with micro-organisms and does not provide any evidence that *T. trimaculatus* has a direct impact on *P. viburni* eggs (Desurmont et al. 2019). Therefore, this mite species has no real potential for biological control against *P. viburni*. The fact that traces of *P. viburni* egg DNA were still present inside the body of some mites despite the absence of impact on *P. viburni* eggs indicates that detection of an organism's DNA within a putative predator does not always equal predation, and underlines the benefits of using an integrative approach combining manipulative experiments and molecular analyses to study predation. The seemingly conclusive results of molecular analyses could have led us to wrongly affirm that *T. trimaculatus* is a predator of *P. viburni* eggs if these data had not been counterbalanced by the negative results of direct measures of egg integrity. Therefore, extreme caution should

always be taken with the interpretation of positive results in molecular analyses attempting to reconstruct trophic links and interaction webs.

This study, which emphasizes both the value and the complexity of the application of qPCR to predation studies in mites, may pave the way for investigations of inconspicuous predator–prey systems and multitrophic networks in general. The stepwise methodology we developed to overcome the pitfalls encountered when applying qPCR to our system, namely detectability problems, body surface contamination, and nonspecific DNA amplification, may easily be adapted to many other systems.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3065/full