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LETTER

Parasitic wasp-associated symbiont affects plant-mediated species interactions between herbivores

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Abstract

Microbial mutualistic symbiosis is increasingly recognised as a hidden driving force in the ecology of plant–insect interactions. Although plant-associated and herbivore-associated symbionts clearly affect interactions between plants and herbivores, the effects of symbionts associated with higher trophic levels has been largely overlooked. At the third-trophic level, parasitic wasps are a common group of insects that can inject symbiotic viruses (polydnaviruses) and venom into their herbivorous hosts to support parasitoid offspring development. Here, we show that such third-trophic level symbionts act in combination with venom to affect plant-mediated interactions by reducing colonisation of subsequent herbivore species. This ecological effect correlated with changes induced by polydnaviruses and venom in caterpillar salivary glands and in plant defence responses to herbivory. Because thousands of parasitoid species are associated with mutualistic symbiotic viruses in an intimate, specific relationship, our findings may represent a novel and widespread ecological phenomenon in plant–insect interactions.

Keywords

Herbivore colonisation, parasitoid, plant–insect interactions, polydnaviruses, tritrophic interactions.

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INTRODUCTION

Mutualistic symbiosis is a widespread phenomenon in nature in which partners associate in an intimate relationship with reciprocal benefits. Microbial symbionts can provide benefits to their associated multicellular eukaryotes that include nutritional effects (Bennett & Moran 2015; Douglas 2015) and protection against natural enemies (Scarborough *et al*. 2005; Gerardo & Parker 2014) or against abiotic stress (Montllor *et al*. 2002; Heyworth & Ferrari 2015). The role of mutualistic symbiosis receives increasing attention as a hidden driving force that mediates networks of interacting species (Sanders *et al*. 2016). Plants form the basis of most terrestrial food webs and interact with a suite of different organisms in nature, including herbivorous and carnivorous insects (Schoonhoven *et al*. 2005). It is now recognised that plants are not alone when interacting with other organisms because microbial symbionts may modulate the strength of plant-insect interactions and consequently affect insect community structuring in plant-based food webs. Plant-associated symbionts such as mycorrhizal fungi affect not only plant growth but also plant defences by inducing systemic resistance towards a wide range of attackers including aboveground herbivores (Pineda *et al*. 2010; Jung *et al*. 2012; Pieterse *et al*. 2014). Herbivore-associated symbionts can play a major role in overcoming plant defences (Frago *et al*. 2012; Zhu *et al*. 2014; Sugio *et al*. 2015). For example symbiotic bacteria associated with oral secretions of Colorado potato beetles can manipulate the physiology of tomato plants to the benefit of their herbivorous hosts leading to evasion of anti-herbivore defences (Chung *et al*. 2013). Carnivorous insects such as parasitic wasps are also known to harbour symbionts (Dorémus *et al*. 2014; Drezen *et al*. 2014); however, the ecological effects of such third-trophic level symbionts have not been investigated so far in a plant-insect context. To unravel the complexity of plant-insect interactions and arms races between plants and herbivores, the involvement of the third-trophic level is crucial because plant traits may enhance top-down herbivore suppression by natural enemies (Price *et al*. 1980). This concept deserves to be extended to studying symbiont-mediated effects on tritrophic interactions.

Since the early 1990s a large body of evidence has shown that plants and carnivores commonly interact via herbivore-induced plant volatiles (HIPVs) which recruit predators and parasitoids of the attacking herbivore (Turlings *et al*. 1990; Vet & Dicke 1992), a phenomenon often described as 'cry for help' (Dicke & Baldwin 2010). Endoparasitoids lay their eggs in the herbivore and the parasitoid larvae consume the herbivore from inside out, often keeping the herbivore alive and regulating its growth during parasitoid larval development (Godfray 1994). As a result, parasitoids themselves may interact with the plant by influencing plant responses to herbivory as a consequence of the parasitisation of the attacking herbivore (Poelman *et al*. 2011; Zhu *et al*. 2015; Kaplan *et al*.

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2016; Ode *et al*. 2016). Parasitoid-mediated induction of plant responses has been shown to change the plant's phenotype with consequences for plant-mediated interactions between early (*Pieris brassicae*) and subsequent (*Plutella xylostella*) herbivore colonisers: for example the moth *P. xylostella* prefers to oviposit on cabbage plants previously infested with unparasitised *P. brassicae* caterpillars compared to plants infested with caterpillars parasitised by *Cotesia glomerata* (Poelman *et al*. 2011). Such parasitoid-mediated induction of plant responses occurs via phenotypic changes in the herbivore's oral secretions (regurgitant and/or saliva) which are known to play a key role in inducing plant defence responses (Poelman *et al*. 2011; Shikano *et al*. 2017); indeed, many elicitors that plants use to counteract herbivore attack have been identified in the oral secretions of caterpillars that come in contact with plant tissues during herbivore feeding (Alborn *et al*. 1997; Mattiacci *et al*. 1995; Bonaventure *et al*. 2011, Bonaventure 2012; Rivera-Vega *et al*. 2017).

The effect of parasitisation of herbivores on plant responses has been assumed to be triggered by the parasitic wasp larvae feeding within the herbivore's body (Poelman *et al*. 2011). However, thousands of parasitoid species inject-specific symbiotic viruses (polydnaviruses = PDVs) into their hosts that manipulate herbivore physiology and immune responses (Pennacchio & Strand 2006; Strand & Burke 2013; Dorémus *et al*. 2014; Chevignon *et al*. 2015). PDVs are unique insect viruses as they can only replicate in the calyx region of the wasp's ovary. Subsequently, viral particles are injected into the host caterpillar during parasitism events, to prevent encapsulation of parasitoid eggs by suppressing the caterpillar's immune response (Edson *et al*. 1981; Shelby & Webb 1999; Strand *et al*. 2006; Webb *et al*. 2006; Lu *et al*. 2010; Burke & Strand 2014). The effect of symbiotic wasp viruses on the ecology of plant–insect interactions has not been investigated (Shikano *et al*. 2017). During oviposition, parasitoids also inject venoms which may synergise the effects of PDVs (Asgari & Rivers 2011; Asgari 2012) and can be required for the expression of PDV genes in the caterpillar (Zhang *et al*. 2004).

Here, we experimentally manipulated the phenotype of herbivores feeding on brassicaceous plants. We isolated parasitoid eggs, venom and calyx fluid (containing PDV particles) from the gregarious parasitoid *Cotesia glomerata* (Fig. S1) and injected these parasitoid-derived components into second-instar *P. brassicae* caterpillars subsequently feeding on wild *Brassica oleracea* plants. We discovered that in the event of parasitism, viral symbionts and venom, but not the parasitoid offspring, influence oviposition preference of *P. xylostella* moths, which often colonise brassicaeous plants after pierid butterflies in the field. We further demonstrate that surgical removal of *P. brassicae* salivary glands knocks down plant-mediated species interactions between parasitised caterpillars and *P. xylostella*. We show that these ecological effects of viral symbionts and venom correlate with changes in gene transcript levels in the caterpillar's salivary glands and in plant defence responses to herbivory. Overall, these results elucidate a new ecological role of third-trophic level symbionts in plant–insect interactions, highlighting a fascinating complexity within terrestrial networks of interacting species.

MATERIALS AND METHODS

Plants and insects

Seeds of the wild *Brassica oleracea* population 'Kimmeridge' (Dorset, UK, 50°360N, 2°070W) were grown in a glasshouse compartment (22 ± 3°C, 50–70% relative humidity and 16:8 h L:D photoperiod). Five-week-old plants were used in the experiments. The herbivores (*P. brassicae* and *P. xylostella*) and parasitoids (*C. glomerata*) were originally collected from field sites near Wageningen University, the Netherlands, and reared on cabbage plants (*B. oleracea* var *gemmifera* cv. Cyrus) in glasshouse compartments (22 ± 1°C, 50–70% relative humidity and 16:8 h L:D photoperiod).

Isolation of polydnavirus particles (PDVs), venom and parasitoid eggs

Calyx fluid (containing the PDV particles) and venom were extracted from *C. glomerata* wasps anaesthetised on ice and dissected in phosphate-buffered saline (PBS) under a light microscope. The venom apparatus (gland and reservoir) and the ovaries were collected separately and pooled in 250 μL PCR tubes. The volume was adjusted with PBS to reach the desired concentration in wasp equivalents (w.e.) as described in Dorémus *et al*. 2013 (e.g. venom apparatus from 30 wasps pooled in 30 μL of PBS for injection of 100 nL containing 0.1 w.e./caterpillar). A concentration of 0.1 w.e. was selected considering that after 10 parasitism events depletion of the parasitoids' egg load can occur (Zhu *et al*. 2015), and we assumed that 1/10 of calyx fluid and venom is injected along with the eggs during a parasitism bout. Venom gland and calyx were disrupted by several passages through a 20 μL micropipette cone. Tubes containing the extracts were centrifuged for 5 min at 2800 G (venom) or for 1 min at 28 G (calyx fluid) and then supernatants containing the venom or calyx extracts were stored on ice until injections into second-instar *P. brassicae* caterpillars (as described below). It has been shown that purification of the virus by centrifugation has similar effects on caterpillar physiology as other purification techniques such as filtration or using a gradient (Beckage *et al*. 1994). Presence of PDV particles in calyx extracts was confirmed under an electron microscope Zeiss EM10CR at 80 kV. For injections with a mixture of venom and calyx fluid, equal volumes of the two extracts were mixed before injection experiments. To isolate mature parasitoid eggs and to minimise contamination with PDV particles, second-instar *P. brassicae* caterpillars were parasitised by *C. glomerata* and rapidly dissected in PBS to recover eggs. The eggs were suspended in 30 μL of PBS in a 250 μL PCR tube, pelleted gently (5 s at 112 G) and washed three times using 30 μL of PBS.

Microinjections in caterpillars

Phosphate-buffered saline solutions with components retrieved from parasitic wasps were injected into L2 *P. brassicae* caterpillars anaesthetised with CO_2 using the NanojectII Auto-Nanoliter Injector (Drummond). In all experiments, 0.1 wasp equivalent of venom, calyx fluid or a mixture of venom and calyx fluid (with or without eggs) dissolved in 100 nL were

injected. Eggs were injected as aliquots of PBS containing approximately 20–40 eggs per 100 nL. We prepared seven different caterpillar treatments to test the effect of each of three component of parasitism individually (eggs, PDVs, venom) and their combined effects in a full factorial design: (1) calyx fluid (containing PDVs); (2) venom; (3) eggs; (4) calyx fluid+ venom; (5) calyx fluid + eggs; (6) venom + eggs; (7) calyx fluid + venom + eggs. The last treatment mimics a natural parasitism event. Two additional treatments were used as controls to test whether the microinjection treatment *per se* affected the interaction of the caterpillars with the food plant: (8) unparasitised caterpillars injected with 100 nL of PBS (negative control) and (9) *C. glomerata* parasitised caterpillars injected with 100 nL of PBS (positive control). After microinjections, the caterpillars that recovered within 2 h were transferred to wild *B. oleracea* plants.

To investigate the effect of parasitoid offspring itself (alone or in combination with PDVs and venom), all nine caterpillar treatments were used in herbivore oviposition preference bioassays (see below). To test if symbiotic wasp viruses (alone or in combination with venom) induce phenotypic changes that mimic parasitised herbivores in a plant-insect perspective, treatments 1, 2, 4, 8 and 9 were used in caterpillar- and plant-gene transcriptional analyses (see below).

Herbivore oviposition preference

Response induced by injected caterpillars

To investigate if PDVs, parasitoid offspring and venom affect subsequent herbivore colonisation, we performed two-choice oviposition preference experiments in which female diamondback moths (*P. xylostella*) were tested for their preferences for *B. oleracea* plants previously induced by *P. brassicae* caterpillars injected with PDVs, venom or eggs over control plants (i.e. induced by PBS-injected caterpillars). We followed the methodology described by Poelman *et al.* (2011) in which each plant was infested with two L2 caterpillars that were allowed to feed for 7–10 days after injection treatments. This time window was sufficient to allow parasitoid egg eclosion and development of larvae within the caterpillars (Sato 1980). Furthermore, the time window we chose for induction is relevant from an ecological perspective as *P. xylostella* commonly colonises brassicaceous plants after *P. brassicae* in The Netherlands (Poelman *et al.* 2010). On the morning of the experiment, we detached the leaves from the plant, placed them in glass vials containing tap water and matched them with a similar-sized leaf of the control treatment. The pair of leaves was placed in a plastic cylinder (diameter 13 cm, height 22 cm) in which a male and female moth were released. The females were allowed to oviposit overnight, and the number of eggs on each leaf was counted the next morning. For each pairwise combination we tested 31–41 replicates.

Response induced by ablated caterpillars

To investigate the effect of caterpillar saliva on plant-mediated oviposition preference by diamondback moths, we first surgically removed salivary glands as described by Musser *et al.* (2006) (see Appendix S1) from unparasitised *P. brassicae* caterpillars (PB) and *Cotesia glomerata*-parasitised caterpillars (CG-PB). We then induced wild *B. oleracea* plants for 24 h with two fifth-instar caterpillars treated as follows: (1) *P. brassicae* caterpillars with intact labial salivary glands (PB-S+); (2) *P. brassicae* caterpillars with ablated labial salivary glands (PB-S-); (3) *C. glomerata*-parasitised *P. brassicae* caterpillars with intact labial salivary glands (PB-CG-S+); (4) *C. glomerata*-parasitised *P. brassicae* caterpillars with ablated labial salivary glands (PB-CG-S-); or (5) plants were left untreated serving as the undamaged control (UD). After induction, we tested the subsequent response of diamondback moths in terms of oviposition preferences. In particular, we asked whether the moth is able to discriminate between: (1) plants induced by unparasitised caterpillars and plants induced by parasitised caterpillars with salivary glands ablated (PB-S- vs PB-CG-S-); (2) plants induced by mock-treated caterpillars or caterpillars with labial salivary glands removed (PB-S+ vs PB-S-; and PB-CG-S+ vs PB-CG-S-); (3) undamaged control plants and plants damaged by ablated unparasitised (UD vs PB-S-) or parasitised (UD vs PB-CG-S-) caterpillars. For each pairwise combination we tested 59–73 replicates.

Leaf damage assessment

To analyse whether oviposition preference by diamondback moths correlated with the amount of leaf damage inflicted by caterpillars in the different plant treatments, we quantified the amount of damage on each leaf that had been used in the oviposition experiments. The leaves were taped onto a white paper sheet and scanned with a RICOH Scanjet MPC4503. The scans were analysed for the size of damaged leaf surface by counting the number of pixels making up the damaged area using Adobe Photoshop-CC 2015.1.2. The number of pixels was converted into cm^2 by comparison with the number of pixels that make up a reference 1-cm^2 square. For each of the different treatments, we then used linear regression models to test the effect of the fixed factor 'leaf area damage' on the response variable 'number of eggs laid'.

Labial salivary gland collection, RNA extraction and real-time qPCR

To investigate whether infection with polydnaviruses (PDVs) (alone or in combination with venom), affects transcriptional responses in the herbivore's salivary glands, we quantified relative transcript levels in the salivary glands of genes encoding for glucose dehydrogenase (GDH) and β-glucosidase precursors (BGPs). We targeted *GDH*, whose encoded product is a member of the superfamily of glucose-methanol-choline oxidoreductases (GMCs) that include enzymes known to suppress plant defences such as the related glucose oxidase (GOX) (Musser *et al.* 2002). We also targeted *BGPs* as their final product, β-glucosidase, is an elicitor of plant responses in brassicaceous plants (Mattiacci *et al.* 1995).

Labial salivary glands, collected from differentially treated caterpillars 7–10 days after injection when the herbivore had reached the fifth-larval instar, were immediately placed into an RNase-free 2.2-mL microfuge tube. Each pair of glands was used as one biological replicate (10–12 replicates were carried out for each treatment). These samples were immediately

frozen in liquid nitrogen and stored at −80°C for RNA isolation. RNA was isolated using the RNeasy kit from Qiagen according to the manufacturer's instructions. 2 μg of total RNA was reverse-transcribed into cDNA using Bio-Rad's iSCRIPT cDNA synthesis kit in a 40 μL reaction volume also according to the manufacturer's instructions. Primers (Table S1) were designed based on sequence information of *Pieris brassicae*. iQ SYBRGreen Supermix (Bio-Rad) was used to perform the real time qPCR reactions in duplicate. The following PCR program was used for all PCR reactions (annealing temperature in Table S1): 95°C, 3 min followed by 40 cycles of 95°C for 10 s, annealing temperatures for 10 s and 72°C for 30 s, with data collection at 72°C. The PCR reactions were followed by a melt curve analysis to check for primer-dimer formation or unspecific PCR products. Calibrated Normalised Relative Quantity values are calculated using the Cq values of the unparasitised caterpillar, calibrating for inter-run variation and normalising using the Cq values of the reference genes Elongation Factor 1α and Ribosomal protein s20 which were selected (using geNorm, Vandesompele *et al*. 2002) out of a total of seven reference genes.

Plant responses to injected caterpillars

To study plant responses to differentially injected herbivores, we quantified relative transcript levels of four genes from *B. oleracea* involved in different signal-transduction pathways underlying induced defence. In the Jasmonic Acid (JA) signal-transduction pathway, we targeted a gene coding for the enzyme lipoxygenase (*BoLOX*) whereas in the Salicylic Acid (SA) signal-transduction pathway we targeted the pathogenesis-related-1 gene (*BoPR1*). In addition, we selected two genes involved in the regulation of biosynthesis of defensive metabolites: *BoPIN* that codes for a protease inhibitor, and *BoMYR* that codes for myrosinase, an enzyme important for the metabolism of brassicaceous-specific secondary compounds (glucosinolate-breakdown products).

To rule out quantitative feeding effects by differential herbivore behaviour due to injection treatments, we standardised the amount of damage per treatment. We punctured 3 tiny holes (≈0.5 mm^2) within an area of 1.5 cm in diameter to the youngest fully expanded leaf of each plant, using a sterile pin needle. After puncturing, 3 μL of freshly collected regurgitant from differently injected *P. brassicae* caterpillars was applied to the tiny holes on these mechanically damaged leaves (1 μL of regurgitant for each hole). Regurgitant of caterpillars was collected from the same individuals used for salivary gland extraction. To obtain enough regurgitant for each treatment, we used a capillary to collect regurgitant from several caterpillars, each regurgitating 1–5 μL (Fatouros *et al*. 2005). As control treatments, we included (1) plants with punctured leaves that were treated with water instead of regurgitant; (2) undamaged plants. We harvested leaf discs (1.5 cm in diameter) 2 h after the treatments had been applied, as such induction method has been shown to effectively mimic true herbivory at this time point (Poelman *et al*. 2011). The leaf discs included the puncture site and were collected by punching as described by Zheng *et al*. (2007). Each leaf disc from an individual plant was immediately placed into an RNase-free 2.2-mL microfuge tube as one biological replicate (10–12 replicates were carried for each treatment). These samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA isolation.

Isolation of plant RNA and qPCR

RNA was isolated using the ISOLATE II Plant RNA kit from Bioline according to the manufacturer's instructions. 2 μg of total RNA was reverse-transcribed into cDNA using Bio-Rad's iSCRIPT cDNA synthesis kit in a 40 μL reaction volume according to the manufacturer's instructions. Primers (Table S2) were designed based on sequence information of the used plants or were designed earlier by Poelman *et al*. (2011). iQ SYBRGreen Supermix (Bio-Rad) was used to perform the real time qPCR reactions in duplicate. The following PCR program was used for all PCR reactions (annealing temperatures in Table S2): 95°C, 3 min followed by 40 cycles of 95°C for 10 s, annealing temperatures for 10 s and 72°C for 30 s, with data collection at 72°C. The PCR reactions were followed by a melt curve analysis to check for primer-dimer formation or unspecific PCR products. Delta-delta Cq values are calculated using the Cq values of the untreated plants and normalising using the Cq values of the reference genes *BTUB* and *ACT2* which were selected (using geNorm, Vandesompele *et al*. 2002) out of a total of six known reference genes.

Statistical analyses

Oviposition preference of diamondback moths (*P. xylostella*) was analysed using Wilcoxon matched-pair signed-rank tests for each of the pairwise treatment comparisons. ANOVA was used to test if transcript levels of caterpillar and plant genes were significantly affected by microinjection treatments. For transcript levels of salivary glands, we tested the overall effect of gene, treatment and the gene×treatment interaction with a two-way ANOVA. When needed, were log-transformed before analyses were carried out and model fit was assessed with residual plots. Post-hoc differences between the treatments were tested using Tukey tests. Data were analysed with R statistical software (R Development Core Team 2013).

RESULTS

Effects of wasp-associated symbiotic viruses and venom on oviposition preference by subsequent herbivores

Leaves induced by PBS-injected/parasitised caterpillars received fewer eggs than leaves induced by caterpillars injected with PBS only (Wilcoxon matched-pairs signed-ranks test, $Z = 3.8239$, $P < 0.001$). Injection of parasitoid eggs alone or in combination with venom or calyx fluid into caterpillars did not trigger any oviposition discrimination by diamondback moths over leaves induced by PBS-injected caterpillars (Fig. 1). In contrast, leaves induced by caterpillars injected with calyx fluid in combination with venom received fewer eggs than leaves induced by PBS-injected caterpillars ($Z = 2.090$, $P = 0.036$) (Fig. 1). A similar oviposition response was observed in diamondback moths when given a preference between leaves

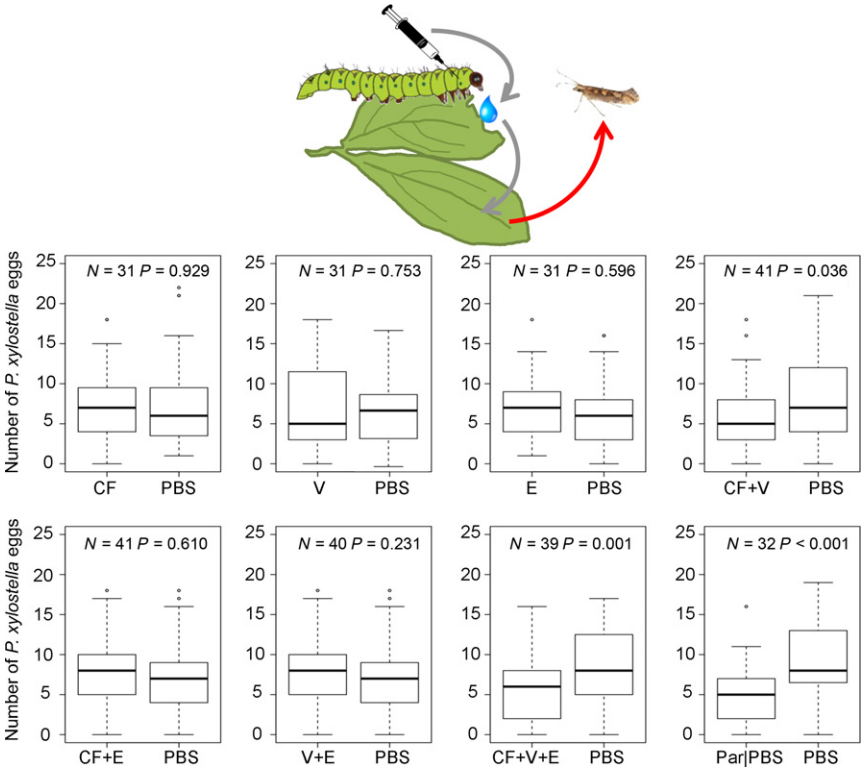

Figure 1 Effect of wasp-associated symbiotic viruses and venom on oviposition preference by subsequent herbivores. Wild *Brassica oleracea* plants were induced for 7–10 days by two second-instar *Pieris brassicae* caterpillars injected with 100 nL of PBS containing 0.1 *Cotesia glomerata* wasp equivalents of: (1) calyx fluid (CF) (containing PDVs); (2) venom (V); (3) eggs (E); (4) calyx fluid + venom; (5) calyx fluid + eggs; (6) venom + eggs; (7) calyx fluid + venom + eggs. Two additional treatments were used: (8) PBS = unparasitized caterpillars injected with 100 nL of PBS and (9) Par|PBS = *C. glomerata* parasitized caterpillars injected with 100 nL of PBS. After induction, treated leaves were offered to diamondback moths in two-choice tests in which a leaf induced by PBS-injected caterpillars was used always as control. The numbers of eggs laid overnight by diamondback moths were used to quantify oviposition preferences. Bold horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points show outliers. Statistical differences within pairwise treatment combinations were carried out with Wilcoxon matched-pair signed-rank test, $P < 0.05$.

induced by PBS-injected caterpillars and those induced by caterpillars in which all parasitoid-derived components (PDVs + venom + eggs) were injected ($Z = 2.538$, $P = 0.001$) (Fig. 1). Although injection of parasitoid-derived components into caterpillars differentially affected the amount of damage inflicted to the plants (Table S3), oviposition preferences of diamondback moths were not correlated with the amount of feeding damage (Table S4).

Effects of surgical removal of caterpillar salivary glands on oviposition preference by subsequent herbivores

In two-choice tests, diamondback moths preferred to oviposit on plants damaged by mock-treated unparasitised *P. brassicae* caterpillars over undamaged control plants (Fig. 2; Wilcoxon's matched-pairs signed-ranks test, $Z = 4.237$, $P < 0.001$). The preference for plants damaged by unparasitised caterpillars over parasitised caterpillars was lost when salivary glands of both caterpillars were surgically removed (Fig. 2, PB-S- vs PB-CG-S-: $Z = -0.331$, $P = 0.741$). Diamondback moths laid fewer eggs on plants induced by *P. brassicae* caterpillars with ablated salivary glands compared to plants induced by mock-treated *P. brassicae* (Fig. 2; PB-S- vs PB-S+: $Z = -3.182$, $P = 0.001$). Furthermore, the moths laid more eggs on plants induced by mock-treated parasitised *P. brassicae* than plants induced by parasitised *P. brassicae* caterpillars with ablated salivary glands (Fig. 2; PB-CG-S+ vs PB-CG-S-: $Z = -2.146$, $P = 0.032$). Finally, the moths did not discriminate between undamaged leaves and leaves induced by unparasitised or parasitised caterpillars with ablated salivary glands (Fig. 2 UD vs PB-S-: $Z = -1.518$, $P = 0.129$; UD vs PB-CG- S-: $Z = -1.337$, $P = 0.181$).

Effects of wasp-associated symbiotic viruses and venom on gene transcription in herbivore salivary glands

Overall, microinjection treatment had a significant main effect on transcript levels (ANOVA: $F_{4,144} = 11.8182$, $P < 0.001$), whereas the main effect of gene identity was not significant ($F_{2,144} = 0.0111$, $P = 0.99$), so the three genes show a similar effect of treatments on transcript level. A significant gene × treatment effect was found ($F_{8,144} = 6.9129$, $P < 0.001$), indicating that the effect of the microinjection treatment is stronger for GDH than for BGP-1 and BGP-2. Injection treatments significantly affected transcript levels of *GDH* in salivary glands (ANOVA: $F_{4,48} = 8.5614$, $P < 0.001$) (Fig. 3a). We found reduced transcript levels in salivary glands from caterpillars injected with calyx fluid + venom and

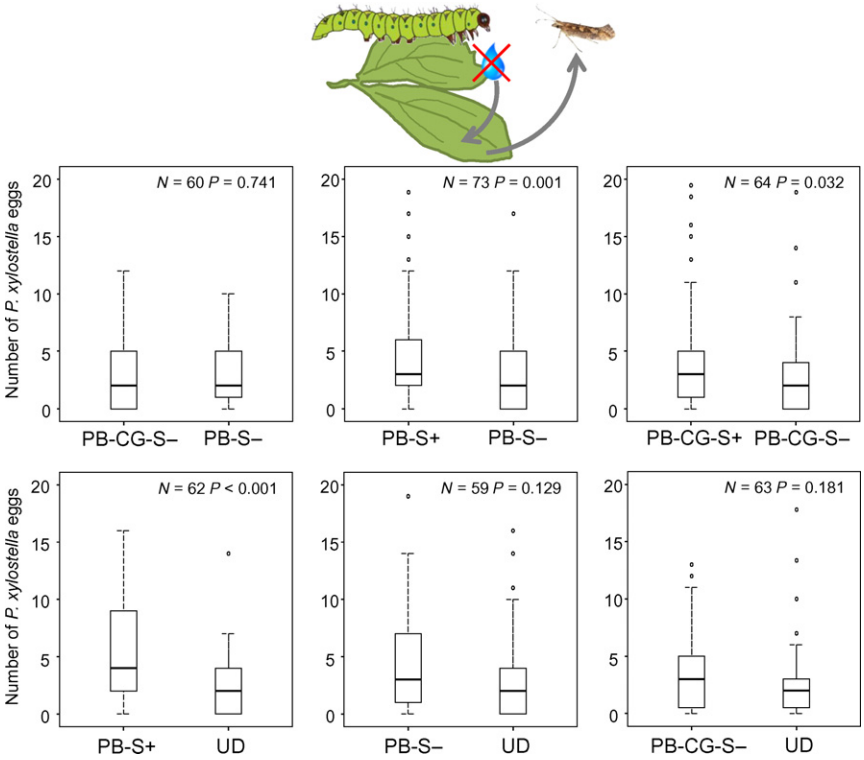

Figure 2 Effect of surgical removal of caterpillar salivary glands on oviposition preference by subsequent herbivores. Wild *Brassica oleracea* plants were induced for 24 h by two fifth-instar *Pieris brassicae* caterpillars whose labial salivary glands had been either surgically removed or left intact. Plants were induced with the following treatments: (1) PB-S+: caterpillars with intact labial salivary glands; (2) PB-S-: caterpillars with ablated labial salivary glands; (3) PB-CG-S+: *C. glomerata* parasitized caterpillars with intact labial salivary glands; (4) PB-CG-S-: *C. glomerata* parasitized caterpillars with ablated labial salivary glands. As controls we used (5) UD: undamaged plants. After induction, treated leaves were offered to diamondback moths in two-choice tests and the numbers of eggs laid overnight were used to quantify oviposition preferences. Bold horizontal lines show medians, boxes contain the 25th–50th percentiles, whiskers show the upper and lower quartiles and points show outliers. Statistical differences within pairwise treatment combinations were carried out with Wilcoxon matched-pair signed-rank tests, $P < 0.05$.

naturally parasitised caterpillars compared to caterpillars injected only with PBS (Fig. 3a). Remarkably, no significant differences were found between transcript levels in PBS-injected/parasitised caterpillars compared with those injected with a combination of calyx fluid plus venom (Fig. 3a). As found for *GDH*, relative transcript levels of *BGP-1* and *BGP-2* were also significantly affected by injection treatments (*BGP-1*: $F_{4,48} = 2.8810$, $P = 0.032$; *BGP-2*: $F_{4,48} = 2.9603$, $P = 0.029$) (Fig. 3b,c). Transcript levels of both *BGP* genes tended to be downregulated in caterpillars injected with calyx fluid +venom as well as in PBS-injected/parasitised caterpillars compared with negative controls (CF+V vs PBS: $P = 0.056$; Par|PBS vs PBS: $P = 0.059$, Tukey test) (Fig. 3b,c). For both *BGP* genes, transcript levels were similar in salivary glands of caterpillars injected with calyx fluid+venom compared with PBS-injected/parasitised caterpillars (Fig. 3b,c).

Effects of wasp-associated symbiotic viruses and venom on transcription of plant-defence-related genes

Regurgitant collected from differentially injected caterpillars affected transcript levels of three plant-defence-related genes (ANOVA, *BoLOX*: $F_{4,55} = 6.5087$, $P < 0.001$; *BoPIN*: $F_{4,55} = 3.9107$, $P = 0.007$; *BoMYR*: $F_{4,55} = 3.3809$, $P = 0.019$), whereas *BoPR1* was not affected ($F_{4,55} = 1.4010$, $P = 0.245$) (Fig. 4). Transcript levels of *BoMYR* were significantly higher in plants treated with regurgitant from caterpillars injected only with PBS over those treated with PBS-injected/parasitised caterpillars or injected with calyx fluid and venom (Fig. 4d). Analyses of transcript levels of *BoMYR* suggest that parasitoid larvae do not play a major role in triggering parasitism-induced responses in plant secondary chemistry (i.e. breakdown of glucosinolates).

DISCUSSION

Plant responses to parasitised caterpillars differ from responses to unparasitised caterpillars and this was assumed to be caused by the parasitoid larvae feeding within the herbivore body (Poelman *et al.* 2011). Here, we challenged this and our results clearly show that calyx fluid containing *C. glomerata* polydnaviruses acts in combination with parasitoid venom to mediate complex ecological interactions at the plant–insect interface which affect the oviposition behaviour of a subsequent herbivore species. These effects correlate with specific changes induced in the herbivore's salivary glands and in plant defence responses to herbivory. Thus, our data show that third-trophic level symbionts play a key ecological role in

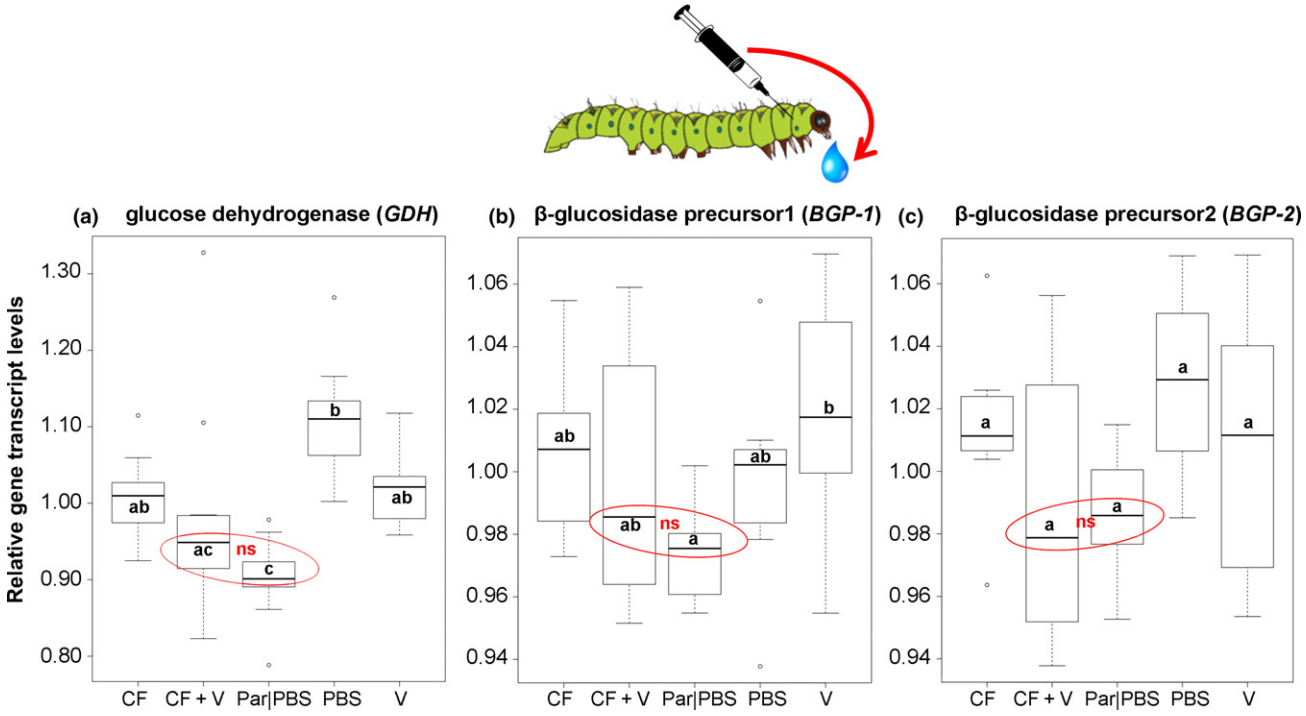

Figure 3 Effect of wasp-associated symbiotic viruses and venom on gene transcript levels in herbivore salivary glands. L2 *Pieris brassicae* caterpillars were injected with 100 nL of PBS containing 0.1 *Cotesia glomerata* wasp equivalents of: (1) calyx fluid (CF) containing polydnaviruses, (2) venom (V) and (3) calyx fluid + venom. Two additional treatments were used as controls: (4) PBS = unparasitized caterpillars injected with 100 nL of PBS and (5) Par|PBS = *C. glomerata* parasitised caterpillars injected with PBS. Relative gene transcript levels of glucose dehydrogenase (GDH) and β-glucosidase precursors (BGPs) were quantified. Bold horizontal lines show medians, boxes contain the 25th – 50th percentiles, whiskers show the upper and lower quartiles and points show outliers. Different letters indicate statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$, $n = 10–12$).

plant–insect interactions, whereas the parasitoid offspring itself is surprisingly not the major driver of parasitism-mediated effects on the induction of plant responses. Although our targeted gene transcription approach shows that virus plus venom affect transcriptional responses in caterpillar salivary gland and in plant tissue, the exact mechanisms underlying the altered behaviour of *P. xylostella* moths remains to be elucidated.

Diamondback moths preferred to oviposit on *B. oleracea* leaves induced by PBS-injected *P. brassicae* caterpillars over leaves induced by caterpillars simultaneously injected with PDVs+venom, isolated from the parasitoid *C. glomerata*. Diamondback moths have been suggested to selectively oviposit on plants previously attacked by unparasitised *P. brassicae* caterpillars because their progeny suffers lower mortality due to reduced larval parasitism in the presence of *P. brassicae* caterpillars (Shiojiri *et al.* 2001, 2002). Localisation of host plants by diamondback moths may be based on volatile organic compounds specific for brassicaceous host plants (i.e. glucosinolate-breakdown products), which are released in high amounts in response to herbivory by unparasitised *P. brassicae* caterpillars (Gols *et al.* 2009). After herbivory by *P. brassicae*, diamondback moths can detect plant phenotypic changes caused by *BoMYR*, which codes for myrosinase, an enzyme important for the metabolism of glucosinolate-breakdown products (Zheng *et al.* 2011). In fact, we found significantly higher transcript levels of this gene in plants induced by PBS-injected (=unparasitised) *P. brassicae* caterpillars over those induced by caterpillars simultaneously injected with PDV and venom or parasitised by *C. glomerata*.

Interestingly, diamondback moths adjust their oviposition preferences based on plant phenotypic changes induced by oral secretions of *P. brassicae* caterpillars simultaneously injected with PDVs and venom. In contrast, changes in quantitative plant traits due to differences in the amount of damage inflicted by differentially injected caterpillars did not affect diamondback moth oviposition discrimination. The key role of herbivore salivary secretions is also supported by evidence that surgical removal of labial salivary glands from *P. brassicae* disrupts oviposition preference of diamondback moths. In fact, plants previously attacked by *P. brassicae* caterpillars with ablated salivary glands are perceived by diamondback moths as undamaged control plants, regardless of the parasitism status of the caterpillars. Due to the importance of salivary glands, it is possible that plant phenotypic changes which affect colonisation by diamondback moths are triggered by changes in elicitors induced by injection of PDVs and venom in *P. brassicae* caterpillars. Indeed, PDVs target several tissues when injected into caterpillars and salivary glands are also specifically infected (Bitra *et al.* 2011) suggesting a direct effect of PDVs on salivary secretions. Although an untargeted transcriptomic approach could reveal the whole pattern of manipulations that PDVs and venom could induce in salivary glands, our targeted approach indicated downregulation of key genes (*BGP-1, BGP-2, GDH*) among which a stronger effect was observed in *GDH* (coding for glucose

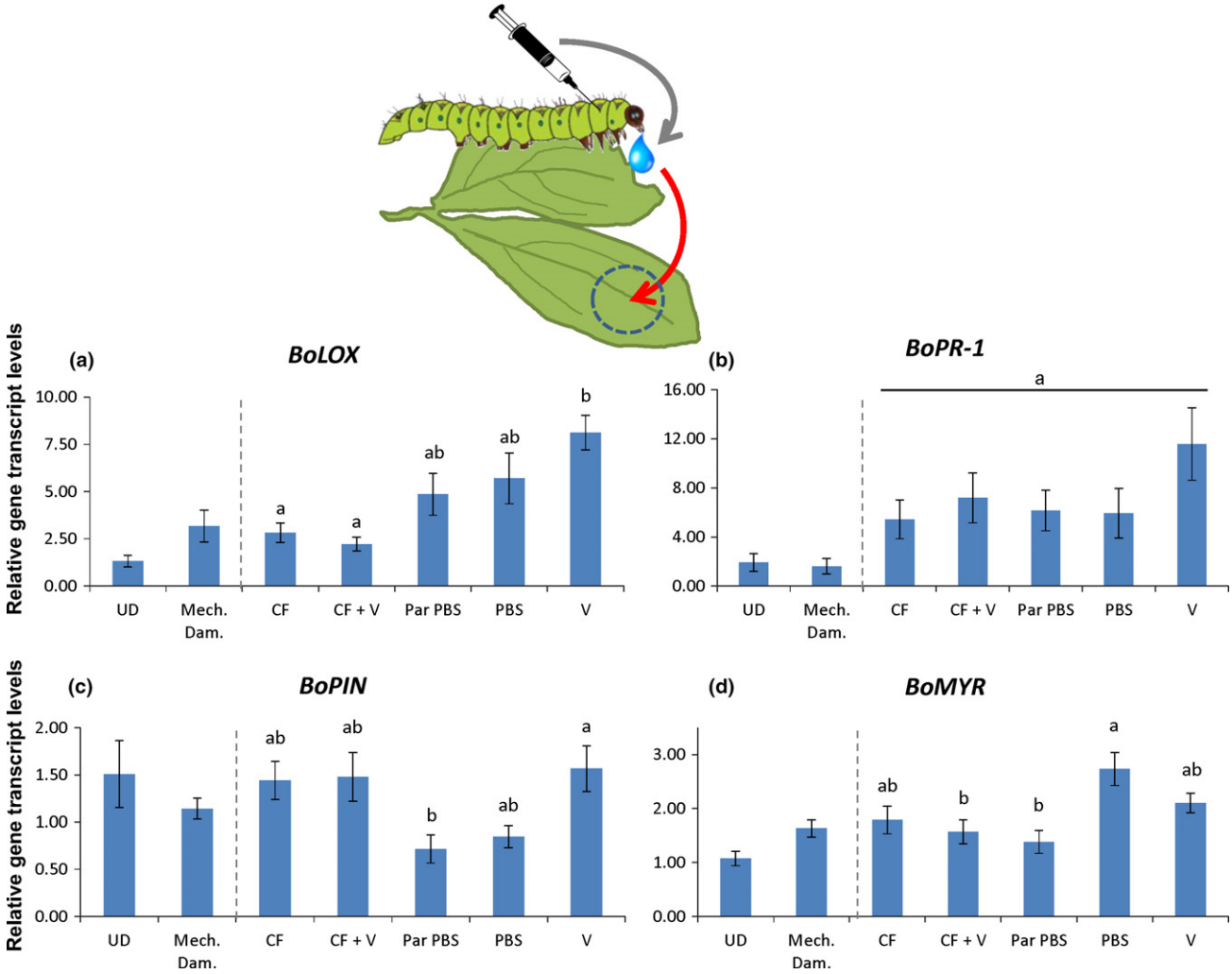

Figure 4 Effect of wasp-associated symbiotic viruses and venom on transcript levels of plant-defence-related genes. Regurgitant was collected from *Pieris brassicae* caterpillars injected with 100 nL of PBS containing 0.1 *Cotesia glomerata* wasp equivalents of: (1) calyx fluid (CF) containing polydnaviruses, (2) venom (V) and (3) calyx fluid + venom. Regurgitant was also collected from (4) PBS = unparasitised caterpillars injected with 100 nL of PBS. (5) Par|PBS = *C. glomerata* parasitised caterpillars injected with PBS. Regurgitant from differently injected caterpillars was applied to mechanically damaged wild *Brassica oleracea* plants and leaf discs containing the damaged area were subsequently harvested 2 h after induction. Relative gene transcript levels of *BoLOX* (a), *BoPR1* (b), *BoPIN* (c) and *BoMYR* (d) were quantified. As additional controls we quantified transcript levels in mechanically damaged plants (Mech. Dam) as well as in undamaged plants (UD). Different letters above bars indicate statistically significant differences (ANOVA followed by Tukey test, $P < 0.05$, $n = 10–12$). Error bars correspond to standard errors.

dehydrogenase), which is closely related to glucose oxidase (GOX), an enzyme known to suppress plant defences in tobacco plants (Musser *et al.* 2002).

From a community perspective, feeding damage by *P. brassicae* caterpillars injected with PDVs and venom could be beneficial for the plant, as it reduced the pressure of colonisation by diamondback moths. Moreover, alterations in herbivore oral secretions mediated by symbionts associated with insects at the third-trophic level could be perceived by the plant as information that the attacking herbivore has been successfully parasitised and thus plants could attenuate defences accordingly. A suggestion that plants could indeed adjust chemical defences in response to parasitism status of the attacking herbivore is found in transcript levels of *BoMYR* which showed similar downregulation patterns after induction with regurgitant collected from caterpillars injected with PDVs and venom compared with PBS-injected/parasitised caterpillars. Changes in transcript levels in response to injection treatments were also found for other plant defence-related genes (*BoLOX*, *BoPIN*) but no differences were found between plant response to unparasitised and parasitised caterpillars, possibly due to the extensive variation in plant traits expressed by the wild population of *B. oleracea* that we used in this study. As expected, no differences were found in transcript levels of *BoPR1* which is a marker for the salicylic acid (SA) pathway and a major regulator of responses to sap-feeding herbivores (de Vos *et al.* 2005; Erb *et al.* 2012; Pieterse *et al.* 2012).

A large body of evidence has shown that PDVs manipulate caterpillar physiology to the benefit of their symbiotic partners (Pennacchio & Strand 2006; Strand & Burke 2013, 2015; Drezen *et al.* 2014). Although the focus of this paper was to

unravel the ecological effects of PDVs on plant-mediated interactions between herbivore species, the hypothesis that PDVs could also manipulate plant physiology to benefit their associated parasitoids merits further investigation. Here, we can speculate that PDVs could increase wasp fitness via changes in food quality cascading along the trophic chain: by reducing chemical defences (i.e. reduction of glucosinolate-breakdown products), *P. brassicae* caterpillars parasitised by *C. glomerata* could have access to plant resources of better nutritional quality which, in turn, could lead to higher quantitative/qualitative resources for the developing parasitoid larvae. Plant nutritional quality is well-known to impact parasitoid fitness via effects on the herbivore host (Ode 2006) and this is especially important in gregarious parasitoids such as *C. glomerata* in which scramble competition may limit the amount of available resources for the developing wasps (Harvey *et al*. 2013). How PDVs and venom act in combination to mimic parasitism-induced effects in a plant-insect context deserves to be investigated in further studies: PDVs affect the physiology of the herbivores to the benefit of their parasitoid partners (Strand & Burke 2013; Dorémus *et al*. 2014; Drezen *et al*. 2014) and it has been shown that the venom often synergises with PDVs (Asgari 2012). Alternatively, PDV genes might be exclusively expressed in the caterpillar body in the presence of the venom as found during *in vitro* experiments in the closely related host–parasitoid system *Pieris rapae* - *C. rubecula* in which the venom is suggested to assist uncoating of viral particles (Zhang *et al*. 2004). In particular, it will be interesting to investigate if PDV genes are expressed in a tissue-specific manner in *P. brassicae* caterpillars parasitised by *C. glomerata* and whether the venom facilitates this process (Bitra *et al*. 2011). In conclusion, our work shows that not only symbionts associated with herbivorous insects can impact plant responses to herbivory (Chung *et al*. 2013; Su *et al*. 2015; Wang *et al*. 2017), but symbionts of carnivorous insects can also play an important role. This finding adds an extra-layer of complexity to the ecology of plant-insect interactions suggesting that consideration of the third-trophic level should be extended to the associated symbionts as well to fully understand how plants cope with herbivores. Studies on host–parasitoid interactions and on plant–herbivore interactions have largely developed independently. Our results unravel exciting connections between these research fields and highlight the importance of placing mutualistic interactions in a community context to unravel sophisticated hidden ecological interactions.

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AUTHORSHIP

AC, ANV, MD and EHP designed the experiments. AC, FZ, JB and PV performed the experiments. AC and FZ analysed data. AC, FZ, ANV, HV, MD and EHP wrote the manuscript.

DATA ACCESSIBILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.gp5km40 (Cusumano et al. 2018).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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