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ORIGINAL ARTICLE

Diurnal feeding as a potential mechanism of osmoregulation in aphids

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Abstract Diurnal variation in phloem sap composition has a strong influence on aphid performance. The sugar-rich phloem sap serves as the sole diet for aphids and a suite of physiological mechanisms and behaviors allow them to tolerate the high osmotic stress. Here, we tested the hypothesis that night-time feeding by aphids is a behavior that takes advantage of the low sugar diet in the night to compensate for osmotic stress incurred while feeding on high sugar diet during the day. Using the electrical penetration graph (EPG) technique, we examined the effects of diurnal rhythm on feeding behaviors of bird cherry-oat aphid (*Rhopalosiphum padi* L.) on wheat. A strong diurnal rhythm in aphids as indicated by the presence of a cyclical pattern of expression in a core clock gene did not impact aphid feeding and similar feeding behaviors were observed during day and night. The major difference observed between day and night feeding was that aphids spent significantly longer time in phloem salivation during the night compared to the day. In contrast, aphid hydration was reduced at the end of the day-time feeding compared to end of the night-time feeding. Gene expression analysis of *R. padi* osmoregulatory genes indicated that sugar breakdown and water transport into the aphid gut was reduced at night. These data suggest that while diurnal variation occurs in phloem sap composition, aphids use night-time feeding to overcome the high osmotic stress incurred while feeding on sugar-rich phloem sap during the day.

Key words bird cherry-oat aphid; diurnal cycle; electrical penetration graph; hydration; osmoregulation; phloem

Introduction

Plants and insects have adapted their growth and development to use diurnal cycling to modify their activities and increase their fitness (Xu *et al*., 2011; Salmela & Weinig, 2019). In plants, photosynthesis, nutrient content, metabolite transport, leaf movement, growth, stomatal opening, and the expression of certain genes are strongly influenced by the time of day (Dodd *et al*., 2005; Greenham & McClung, 2015). In insects, diurnal rhythm has a strong influence on activities such as locomotion (Suzuki & Hori, 2014), mating, and oviposition (Eisenbach & Mittler, 1980), molting and honeydew excretion (Joschinski *et al*., 2016) that directly affect survivorship and reproductive success. Behaviors such as host finding (Arakaki, 1989; Narayandas & Alyokhin, 2006), pheromone release in mate finding (Thieme & Dixon, 1996), and oviposition (Hodgson & Lane, 1981) are especially common during the day-time.

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Diurnal variation in plant nutrient composition has a strong impact on herbivorous insect feeding and performance. In several plant species, variation in sugar and amino acid content of phloem sap composition has been documented over a 24-h period (Hayashi & Chino, 1986; Winter *et al*., 1992; Caputo & Barneix, 1999; Gattolin *et al*., 2008; Taylor *et al*., 2012). Specifically, lower concentrations and/or flux of sucrose has been observed during the night. As important agricultural pests, aphids have been intensively studied in terms of the relationship between phloem sap composition and performance. Aphids rely on nutrient-rich phloem sap as their sole source of nutrition (Douglas, 2006) and variations in phloem sap composition from the diurnal cycle to the season strongly influence aphid performance and behavior (Douglas, 1993; Karley *et al*., 2002; Cao *et al*., 2018). For example, aphids had higher survival, reduced development time and greater fecundity on pretuber filling potato plants compared to tuber filling plants, which was correlated with changes in the phloem amino acid composition and with the increase in total leaf C : N ratio (Karley *et al*., 2002, 2003). Indeed, poor aphid performance has been recorded on developmentally mature plants which are nutritionally inferior compared to younger plants for several aphid species (van Emden & Bashford, 1971; Williams, 1995; Kazemi & van Emden, 1992). Most evidence of effects of diurnal variation on aphid feeding behavior and performance come from records of honeydew production. Honeydew production has been used as a proxy for aphid feeding behavior, and the deposition of honeydew has been found to be higher during the day as compared to the night suggesting reduced aphid feeding during the night (Maxwell & Palinter, 1959; Cull & Emden, 1977; Gomez *et al*., 2006; Taylor *et al*., 2012). However, to date, there is no direct evidence that a diurnal pattern exists in aphid feeding behavior.

The nutrient-rich phloem sap provides an abundant source of carbon and nitrogen that is relatively free of toxins and feeding deterrents (Turgeon & Wolf, 2009). However, in order to exploit phloem sap as a food source, aphids must overcome the high osmotic potential, caused primarily by high sucrose concentrations. Ingesting phloem sap causes a high osmotic gradient between gut contents and body fluids resulting in transfer of water from the body fluids to the gut (Douglas, 2006; Dinant *et al*., 2010). A suite of physiological mechanisms and behaviors allow aphids to tolerate the high osmotic stress and exploit sugar-rich phloem sap as a food source. First, ingested sucrose is hydrolyzed, fructose is assimilated, and glucose molecules are polymerized into oligosaccharides by a gut sucrase (Cristofoletti *et al*., 2003; Price *et al*., 2007). Second, water cycling from the distal to the proximal regions of the gut contributes to osmoregulation and the high flux of water is mediated by membrane-associated aquaporins (Shakesby *et al*., 2009). Third, dilution of the gut contents from water cycling results in the production and excretion of large amounts of honeydew (Wilkinson *et al*., 1997), which is iso-osmotic with the hemolymph. And lastly, aphids frequently ingest xylem sap which has low osmolality to help regulate osmotic potential (Pompon *et al*., 2010; Pompon *et al*., 2011). Given that diurnal variation exists in phloem sap composition, it is plausible that night-time feeding is another factor contributing to the regulation of osmotic potential in aphids. Cull and Emden (1977) suggest that aphids compensate for the high osmotic stress that they incur during day-time feeding by also feeding on more dilute sap in the plant at night.

In the current study, we investigated the effects of diurnal cycles on feeding behaviors of bird cherry-oat aphid (*Rhopalosiphum padi* L.), a major pest of cereals worldwide and the principal vector of many viruses including the barley yellow dwarf virus and the cereal yellow dwarf virus (McPherson *et al*., 1986). Using the electrical penetration graph (EPG) technique, we monitored aphid feeding behavior during the day and night. Aphid hydration was measured at the end of the day and night to correlate feeding with concomitant changes in body water content. Further, we analyzed gene expression of the circadian core clock gene, *timeless* (*TIM*), and three osmoregulatory genes, *sucrase 1* (*SUC1*), *sugar transporter 4* (*ST4*) and *aquaporin 1* (*AQP1*), to examine whether diurnal feeding may be another potential mechanism of osmoregulation in aphids.

Materials and methods

Plant and insect source

Bird cherry-oat aphids (*R. padi*) colony was initiated from aphids that were collected from Riley county, KS from a field at the Rocky Ford Experimental Station, Manhattan, KS and has been maintained in the laboratory since August 2016. The aphid colony was reared on winter wheat variety Coker 9553 (AgriPro®) on Miracle-Gro Potting mix at 50% relative humidity, temperature of 24 $\pm$ 1 °C and a photoperiod of 16 : 8 (L : D) hours. Plants were fertilized once every two weeks using Miracle-Gro® All Purpose Plant food as per label instructions. All experiments were performed on wheat plants that were 14–21 d old (Zadoks stage, Z1.2) (Zadoks *et al*., 1974).

Table 1 Candidate *Rhopalosiphum padi* genes for circadian clock and osmoregulation.

R. padi[†]		*Acrythosiphon pisum*[‡]	
Candidate gene	APHIDBASE unique name	APHIDBASE unique name	Identity
AQUAPORIN 1	Rpa10052.t1	ACYPI006387	91%
GUT SUCRASE 1	Rpa13848.t1	ACYPI000002	91%
GUT SUCROSE TRANSPORTER	Rpa14827.t1	ACYPI001980	92%
TIMELESS	Rpa10053.t1	ACYPI36439	90%

[†]The unique name for the *R. padi* genes is derived from the Whole Genome Assembly v2 and Structural Annotation v2 situated at the Bioinformatics Platform for Agroecosystem Arthropods (BIPAA).

[‡]The unique name for the *A. pisum* genes is derived from the Whole Genome Assembly v3.0 and Structural Annotation OGS3.0 situated at the Bioinformatics Platform for Agroecosystem Arthropods (BIPAA).

Electrical penetration graph (EPG) analysis

Adult *R. padi* feeding behavior on wheat plants was monitored using the electrical penetration graph technique (EPG) on a GIGA 8 complete system (EPG Systems, Wageningen, the Netherlands) (Tjallingii & Esch, 1993) as per Nalam *et al*. (2018). Adult aphids were starved for 1 h prior to wiring. After wiring of aphids was completed, eight wheat plants at Zadoks stage, Z1.2, were placed into a Faraday cage. The wired plant electrodes were then placed into the soil, and insect probes adjusted allowing for contact between the plant surface and the insect. Aphids were allowed to feed for 8 h, while the feeding behavior was recorded. To determine end of night or day feeding behaviors, aphids were allowed to begin feeding at approximately 3 h after lights were turned on (at 05.00) or *Zeitgeber* time 3 (ZT3). To record end of day or night feeding behaviors, aphids were allowed to begin feeding at approximately ZT16 or 21.00 when the lights were turned off. Day versus night feeding behavior was compared by analyzing the amount of time spent in each of the four main phases: pathway phase (PP), nonprobing phase (NP), sieve element phase (SEP), and xylem phase (G). The subphases within SEP that indicate phloem salivation (E1) and phloem ingestion (E2) were also analyzed. Additionally, time to 1st probe, total number of probes, and the number of potential drops, were parameters that provided an indication of aphid health (Martin *et al*., 1997). Measured parameters that provide an indication of phloem acceptability and plant defense response include, the potential E2 index, number of E1 and E2 waveforms, time spent in E1 and E2, as well as percent time spent in E2 that was greater than 10 min (Van Helden & Tjallingii, 2000). EPG waveforms and results were analyzed using Stylet+ software (EPG Systems, Wageningen, the Netherlands). The experiment was repeated until a minimum of 20 replicates were obtained for each treatment. A recording was not considered as a replicate if aphids spent greater than 70% of the recording time in nonprobing + derailed stylet + xylem activities. There were 24 replicates for aphids feeding during the day and 20 replicates for aphids feeding during the night. The data were rank transformed and differences between means were determined using ANOVA (Nalam *et al*., 2018). The comparison of proportions was performed using the "N-1" chi-squared test (Campbell, 2007, Richardson, 2011).

Identification of Rhopalosiphum padi *candidate genes*

Candidate osmoregulatory (aquaporin, gut sucrase, sucrose transporter) and diurnal rhythm (*timeless*) genes in *R. padi* were identified using annotated genes from the pea aphid (*Acyrthosiphon pisum*). The *R. padi* homologs of *sucrase 1* (*RpSUC1*), *sugar transporter 4* (*RpST4*) and *aquaporin 1* (*RpAQP1*) genes were identified using sequences for these genes from *A. pisum*, which have been empirically validated (Price *et al*., 2007; Shakesby *et al*., 2009; Price & Gatehouse, 2014; Tzin *et al*., 2015). Coding sequences for each target gene were used to query the *R. padi* genome assembly (v1.0) available on AphidBase (https://bipaa.genouest.org/is/aphidbase/). *R. padi* nucleotide sequences having the highest identity to *A. pisum* annotated target genes (Table 1) were used to design primers for gene expression analyses in *R. padi* (Table S1). The target gene identities of gut *sucrase 1* (*RpSUC1*) and gut *sugar transporter 4* (*RpST4*) were confirmed using alignments with annotated osmoregulatory target genes identified in green peach aphid (Tzin *et al*., 2015) as a way to ensure that the *R. padi* homologs had a

Table 2 Feeding behavior of *Rhopalosiphum padi* during the day and night.

Parameter		Day $n = 23$ Mean ± SEM	Night $n = 20$ Mean ± SEM	F-value (1, 41)	P value
Probing behavior					
Number of nonprobes (np)	(count)	6.9 ± 0.9	8.9 ± 1.5	0.66	0.420
Time to 1st probe	(min)	6.8 ± 2.1	6.3 ± 2.1	0.32	0.574
Number of probes	(count)	6.9 ± 0.9	9.0 ± 1.5	1.00	0.323
Number of potential drops (pd)	(count)	64.3 ± 6.8	61.9 ± 11.0	0.43	0.517
Sum of pd	(min)	5.5 ± 0.6	4.8 ± 0.8	0.81	0.374
Sieve element phase (SEP)					
Aphids with SEP	(count) (%)	22/23 (96%)	20/20 (100%)		0.377[†]
Number of E1 Waveforms	(count)	6.7 ± 0.8	5.6 ± 0.8	0.91	0.346
Time to 1st E1	(min)	89.1 ± 19.9	54.0 ± 10.7	1.63	0.209
Total duration of E1 followed by E2	(min)	14.5 ± 3.6	42.6 ± 11.9	7.85	***0.008***
Number of E2 Waveforms	(count)	3.4 ± 0.4	2.1 ± 0.3	3.36	***0.046***
Time to 1st E2	(min)	130.6 ± 27.4	138.5 ± 25.7	0.21	0.649
Potential E2 Index	%	41.2 ± 6.8	59.3 ± 8.7	4.37	***0.043***
% E2 >10 min	%	44.8 ± 7.0	72.1 ± 7.2	7.16	***0.011***
Xylem feeding (G)					
Aphids with Xylem phase	(count) (%)	22/23 (96%)	15/20 (75%)		***0.045*** [†]
Number of G	(count)	2.9 ± 0.5	2.1 ± 0.6	3.54	0.057
Time spent in G	(min)	92.2 ± 20.4	81.1 ± 12.9	0.24	0.627

Note. Values in bold are significantly different between treatments at $P < 0.05$.

[†]Refers to the χ^2 comparison of proportions was performed using the "N-1" chi-squared test.

high probability of being expressed in aphids guts alone and not in the whole body.

Analysis of gene expression by real-time-quantitative PCR

The expression of the circadian clock gene and three osmoregulatory genes in *R. padi* was quantified using real-time quantitative PCR (RT-qPCR). Twenty late instar (third and fourth instars) and adult aphids were collected from host plants from three biological replicates or experiments at each time-point. Aphids were sampled at eight different time points along the day/night cycles starting at 3 h after the lights came on, or 3 (ZT3), and collected at 6-h intervals (ZT9, ZT15, and ZT21) over 2 d. Total RNA was extracted from aphids homogenized in Trisure® (Bioline Meridian Bioscience, Memphis, TN, USA) using a Direct-zol® RNA Purification Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's recommendations. Complementary DNA (cDNA) was synthesized from 2 μg of RNA from each sample using the Verso® cDNA Synthesis Kit (Thermo Fisher Scientific, Grand Island, NY, USA). Primers specific to each target gene (Table 2) were tested using MyTaq^{TM} Red DNA Polymerase (Bioline Meridian Bioscience, Memphis, TN, USA) with the following PCR protocol: Initial denature 95 °C for 1 min and 25 cycles of denaturation at 95 °C for 15 s; anneal at primer specified temperature for 15 s; extension at 72 °C for 10 s followed by a final extension at 72 °C for 3 min. PCR products were run on 1% agarose gels to verify target gene fragment size (data not shown). Real-Time quantitative PCR was carried out using iTaq^{TM} Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) with triplicate technical replicates for each sample on a QuantStudio® 3 Real-Time PCR System (Applied Biosystems®, Thermo Fisher Scientific, Grand Island, NY, USA) using the following protocol: Initial denaturation at 95 °C for 30 s and 40 cycles of denaturation at 95 °C for 15 s; anneal, extend, and plate read at 60 °C for 1 min followed by a melt curve of PCR product to verify amplification of specific target. The gene *actin* (*RpACT*) was used as a reference gene. Mean relative expression for each sample was calculated using normalized relative quantities as per Hellemans *et al*. (2007). Primer efficiencies for each primer pair and RT-qPCR assays were calculated using the LinRegPCR program

(Ruijter *et al*., 2009) for analysis of RT-qPCR data. Differences in gene expression between the different time points were tested for statistical significance using ANOVA, and *post hoc* comparison of means was performed using Tukey tests in Minitab 19® (State College, Pennsylvania, USA). In instances where ANOVA tests indicated significant differences in expression between the ZTs for the different genes, a COSINOR analysis (Refinetti *et al*., 2007; Molcan, 2019) was performed to test for circadian rhythmicity and to obtain the parameters that define the rhythm (MESOR, amplitude and acrophase).

Measurement of aphid hydration

Adult *R. padi* were collected from wheat plants maintained in the greenhouse under a 16 : 8 (L : D) regime. Three sets of 30 aphids were collected at the end of the night/beginning of the day and at the end of the day/beginning of the night. To estimate body water content, 30 adult aphids from two different wheat plants were collected and weighed immediately. Dry weights of the aphids were obtained after drying the aphids at 55 °C for 8 h until no change in aphid weight was observed. The body water content of the aphids was determined by subtracting the dry weight from the fresh weight. Percent moisture of the aphids at the two time points was calculated by dividing the body water content by the fresh weight of the aphids. The experiment was replicated three times. Differences in hydration status between the two treatment conditions was tested for statistical significance using Student's *t*-test in Minitab 19®

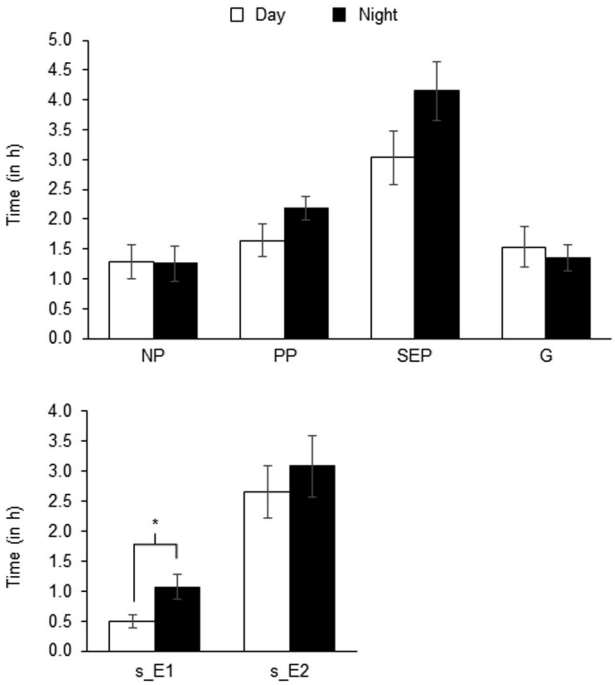

Fig. 1 Electrical penetration graph analysis of *R. padi* during the day and night.
Top Panel. Time spent by the aphid in nonprobing (NP), pathway phase (PP), sieve-element phase (SEP) and the xylem phase (G) in 8 h of recording time during the day and night.
Bottom panel. Total time spent by the aphid in the salivation (s_E1) and phloem sap ingestion (s_E2) in an 8 h recording time. Each bar represents that mean ± standard error of mean. For day-time feeding, $n = 23$ and for night-time feeding, $n = 20$. Asterisks represent values that are significantly different ($P < 0.05$).

Results

Aphids exhibit similar feeding behaviors during day and night

In the current study, we used the electrical penetration graph technique to determine the impact of the diurnal rhythm on aphid feeding behaviors. Overall, the major aphid feeding phases, nonprobing (NP), pathway (PP), sieve element (SEP), and xylem phase (G) did not vary significantly between day and night feeding (Fig. 1, Table 2). However, differences were observed within the sieve element phase with respect to salivation and in the proportion of aphids feeding from the xylem between day and night feeding (Fig. 1, bottom panel, Table 2).

Pathway phase (PP) The probing activity of aphids is usually related to the epidermis and mesophyll cells of the plant and provides an indication of not only plant acceptability but also the health of the aphid. With respect to day-time versus night-time feeding aphids, no significant differences were observed in various aphid probing behaviors (Table 2). The time spent in nonprobing, the total number of nonprobing periods, and in the time to first probe were not significantly different (Table 2). Probing-related activities such as the total number of probes and the total time spent in probing were similar in aphids feeding during day and night (Table 2). Within PP, no significant differences were observed in the number of potential drops (pd) and total time spent in pd (Table 2).

Sieve element phase (SEP) The SEP refers to the phase when the aphid stylet is located inside the phloem specifically the sieve elements (Prado & Tjallingii, 1994). In the SEP, salivation (waveform E1) always precedes phloem sap ingestion (E2) and sustained phloem

sap ingestion (sE2; E2 >10 min). Our data show that aphids were equally successful in finding the sieve elements during day and night (96% and 100%, respectively) (Table 2). Further, a diurnal rhythm was not observed in the total time spent by the aphids in SEP (Table 2). In contrast, there were significant differences in salivation (E1) and phloem sap ingestion (E2) patterns between day and night feeding. On average, aphids spent significantly longer time in E1 (either E1 alone or E1 followed by E2) when feeding during the night as compared to day-time feeding ($F_{1,41} = 7.85$; $P = 0.008$) (Fig. 1). Although no significant differences were observed in the time to first E1 or E2 (Table 2), aphids feeding in the night spent significantly longer times in salivation before committed phloem ingestion (Fig. 1, bottom panel). The potential E2 index, that is, the percentage of time spent in E2 after the first sustained E2 and the mean duration of E2 periods both reflect the persistence of phloem feeding (van Helden & Tjallingii, 1993); low values for these parameters are related to phloem-located factors of resistance. Aphids feeding during the day showed reduced potential E2 index ($F_{1,41} = 4.37$, $P = 0.043$) compared to aphids feeding during the night (Table 2). Further, aphids feeding during the night spent a significantly longer percentage of time in sustained E2 (sE2) compared to aphids feeding during the day ($F_{1,41} = 7.16$, $P = 0.011$) (Table 2).

Xylem phase (G) A significantly lower proportion of *R. padi* displayed G when feeding during the night (96% during the day and 75% during the night, $\chi^2 = 3.881$; $P = 0.045$). However, among the aphids that displayed G, no significant differences were observed in the number of G waveforms and in the total time spent in G during the day and night (Fig 1, Table 2).

Diurnal pattern in aphid hydration status

Based on the results from the EPG experiment, we sought to determine if night-feeding influenced aphid hydration status. Adult *R. padi* collected at the end of the day/beginning of the night exhibited significantly lower hydration or percent moisture compared to aphids collected at the end of the night/beginning of the day ($F_{1,16} = 14.35$, $P = 0.003$, Fig. 2). In aphids collected at the end of the night, the percent moisture in the adult aphids was 3.8% higher than the aphid measured at the end of the day. This result corroborates the increased phloem ingestion observed during night-time feeding (Table 2).

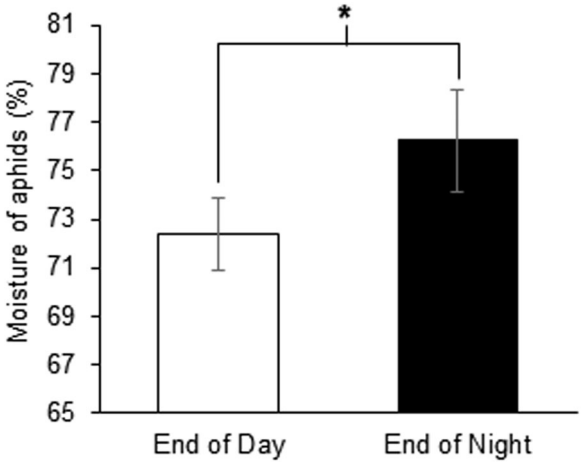

Fig. 2 Aphid hydration at the end of each feeding period. Percent moisture in aphids sampled at the end of the day and at the end of the night. Each bar represents the percentage of water content (mean ± standard error of mean; n = nine groups of 30 aphids) in aphids. Asterisk represents values that are significantly different ($P < 0.05$; Student's t-test).

Diurnal pattern in aphid clock gene expression

To confirm that aphids used in our study display a robust cycling of the internal clock and a diurnal rhythm, the expression of the *R. padi* core clock gene *timeless* (*RpTIM*) was monitored using RT-qPCR analysis. In pea aphids (*A. pisum*), *timeless* was shown to be a part of a core clock response and displays a significant circadian rhythm in aphids reared under long day conditions (Barberà *et al*., 2017). The expression of the *R. padi* aphid homolog of the *timeless* gene (Rpa10052.t1) gradually increased during the day and peak expression was observed at the end of 15 h or ZT15 on two consecutive days (Fig. 3). The expression profile of *RpTIM* indicates that *R. padi* possess a diurnal rhythm when reared under 16 : 8 h light: dark regimen under laboratory conditions.

Diurnal pattern in aphid osmoregulatory genes

To examine the impact of day and night feeding on osmoregulation in aphids, expression of *sucrase 1*, *sugar transporter 4* and *aquaporin 1* genes were monitored in *R. padi* at 6 h intervals over a period of 2 d (Fig. 4, Table S2). Analysis of the expression profile of *RpSUC1* indicated a cyclical pattern of expression over the 2-d period, with the peak between ZT9 and ZT15 and decline from ZT21 to ZT3 (Fig. 4). The water transporter, *RpAQP1* exhibited a similar pattern, with expression peaking at ZT9

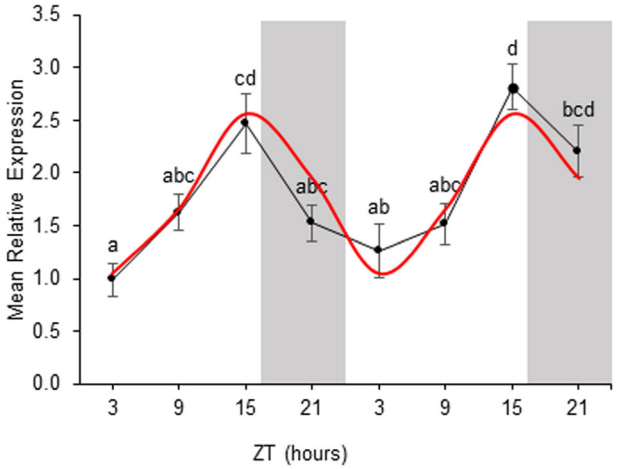

Fig. 3 Expression profile of the circadian clock core gene, *timeless (TIM)*.
R. padi were reared in long day conditions (16 L: 8 D). The mean relative expression of three replicates ± SEM are plotted at 6 h intervals over 2 d. Means that do not share a letter are significantly different. The scotophase of the photoperiods is indicated as grey background. ZT, Zeitgeber time. The circadian rhythm estimated by the COSINOR software for *TIM* is represented by the red curves. *TIM* exhibits a significant circadian rhythm (Table S2).

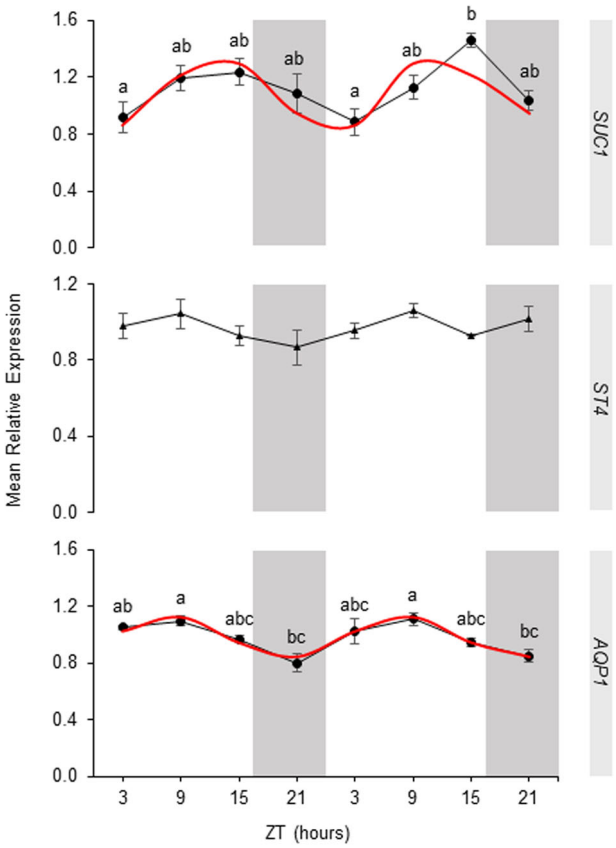

Fig. 4 Expression profile of genes that function in osmoregulation in aphid guts, *sucrase 1* (*SUC1*), *sugar transporter 4 (ST4)* and *aquaporin 1* (*AQP1*).
R. padi reared in long day conditions (16 L : 8 D). The mean relative expression of three replicates ± standard error of mean are plotted at 6 h intervals over 2 d. Means that do not share a letter are significantly different. The dark phase of the photoperiods is indicated as grey background ranging from ZT 16 to ZT 24. The circadian rhythm estimated by the COSINOR software for the genes that show a significant difference between the ZTs in ANOVA tests is represented by the red curves. *SUC1*and *AQP1* exhibit a significant circadian rhythm (Table S2).

and decreasing thereafter (Fig. 4). No significant differences in expression were observed for any of the time points for *RpST4* (Fig. 4).

Discussion

Aphids overcome the osmotic challenge posed by the high sugar content of the phloem sap using a combination of morphological, behavioral, and osmoregulatory traits (Fig. 5). Previous research showed that aphid honeydew production increased during the day compared to night-time feeding, which parallels changes in concentrations/fluxes of sugars and amino acids in the phloem sap. However, it is still unclear whether aphids exhibit diurnal patterns in feeding behaviors is still unclear. Our analysis of aphid feeding using the EPG technique indicated that aphid feeding is unaffected by the time of day. In contrast, aphid hydration status was reduced at the end of the day compared to aphids collected at the end of the night. We hypothesize that continued night-time feeding allowed aphids to take advantage of the more dilute phloem to reach higher levels of hydration compared to day-time feeding. Further, gene expression analysis indicated that sugar breakdown and water transport into the aphid gut was reduced at night-time, providing further evidence that phloem sap is dilute at night. Taken together, these data suggest that night-time feeding in aphids coupled with changes in aphid hydration and in osmoregulatory genes allows aphids to rehydrate and achieve osmoregulation (Fig. 5).

The circadian clock in aphids drives diurnal patterns in various behaviors (Narayandas & Alyokhin, 2006; Joschinski *et al*., 2016; Barberà *et al*., 2017) with activities such as host and mate finding being especially common during the day-time (Arakaki, 1989; Thieme & Dixon, 1996). In our study, we followed the expression pattern of the *timeless* gene since this gene is one of the

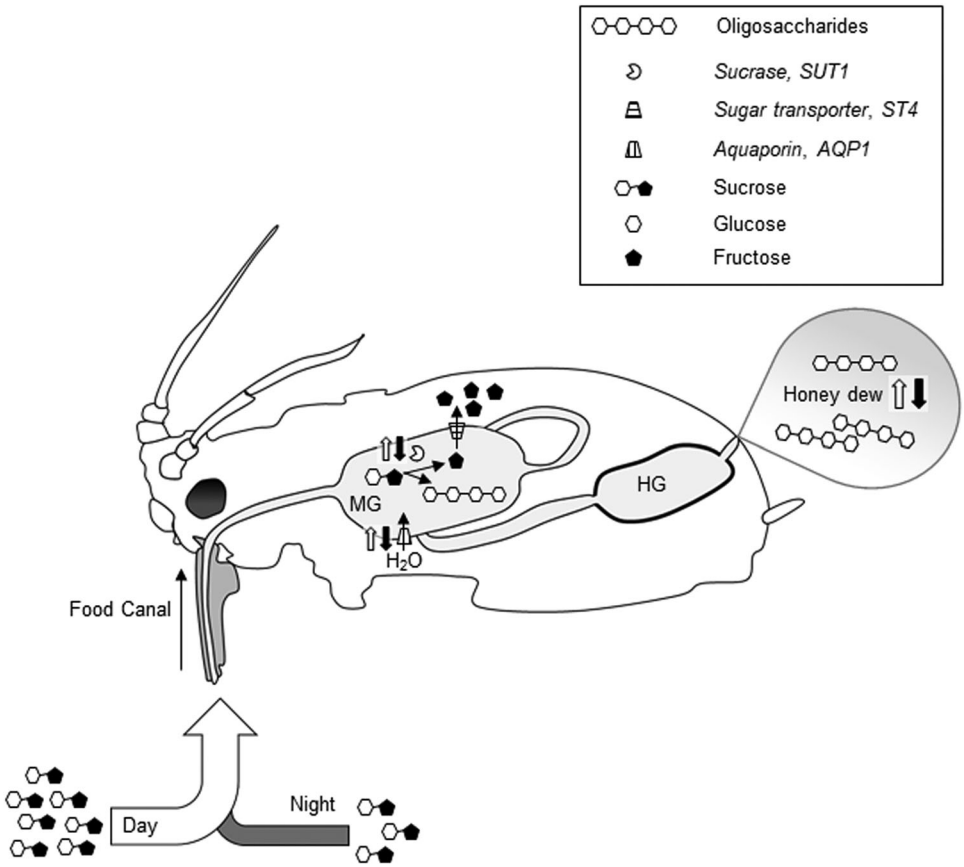

Fig. 5 Conceptual model showing the various mechanisms of osmoregulation in aphids.
Aphids possess several mechanisms to counter the high osmotic pressure generated by sugars in the plant phloem sap. Anatomically, the arrangement of the midgut (MG) and hindgut (HG) allows for the rapid transfer of water between the proximal and distal regions. This transfer of water and the equilibration of the osmotic pressure between the hemolymph and the gut is facilitated by the gene *aquaporin 1* (*AQP1*). In the gut lumen, a sucrase-transglucosidase coded by the gene *sucrase 1* (*SUC1*) breaks down sucrose to glucose and fructose. *SUC1* combines glucose monosaccharides to generate oligosaccharides that are excreted as honeydew. Fructose in the gut lumen is rapidly transported to the hemolymph by the gene *sugar transporter 4* (*ST4*). Additionally, aphids ingest xylem sap which has a diluted composition relative to phloem sap to replenish their water balance. The empty and filled arrows indicate relative amount of honey dew produced or the level of gene expression observed during the day (empty arrow) and night (filled arrow).

five core clock genes and plays an integral role in the functioning of the circadian clock (Hardin, 2011; Barberà *et al*., 2017). The cyclical expression pattern of *timeless* in *R. padi* indicates the presence of a strong diurnal rhythm and this did not impact aphid feeding during the night. The EPG recordings of adult *R. padi* feeding during the day and night-time displayed the same feeding patterns on the host plant which include PP, NP, SEP and G phases. Additionally, no impact of time of day was observed on parameters that provide an indication of aphid health: time to 1st probe, total number of probes and the number of potential drops. Our data provide strong evidence that irrespective of the time of day aphids are able to feed from the host plant. By using the rate of honeydew production as a proxy for aphid feeding, Taylor *et al*. (2012) show that green peach aphid (*Myzus persicae*) and potato aphid (*Macrosiphum euphorbiae*) display a similar behavior when feeding on potato. Ni and Quisenberry (1997) used the EPG technique to show that Russian wheat aphids (*Diuraphis noxia*) also fed on wheat during the night. The ability to feed during the night is not limited to aphids alone but is also observed in other phloem feeding insects such as the mirid bugs, the rice leaf bug (*Trigonotylus caelestialium*) and sorghum leaf bug (*Stenotus rubrovittatus*) on rice (Suzuki & Hori, 2014) and the Asian citrus psyllid (*Diaphorina citri*) on citrus (Serikawa, 2011). These results suggest the universal occurrence of night-time feeding in a diverse class of

phloem feeding insects. However, this may not be the case with all aphid species and all behaviors (Arakaki, 1989; Losey & Denno, 1998).

Our data did not show diurnal pattern on the total time spent in the SEP; however, a significant difference was detected between day and night feeding by *R. padi* with respect to the subpatterns found within the SEP, phloem salivation (E1) and phloem ingestion (E2). Aphids salivated (E1) for approximately two times longer during the night. The salivation phase always precedes phloem ingestion and during this phase aphids are secreting watery saliva into the sieve elements. Watery saliva contains several proteins, some of which have well-known biochemical activity that could either act as elicitors of plant defense or facilitate infestation by suppressing defenses and activating the transport of sugars (reviewed in Nalam *et al*., 2019). Longer salivation periods may therefore be necessary during the night to suppress plant defense traits activated as part of the plants own diurnal rhythm (Goodspeed *et al*., 2012; Jander, 2012). Whether plant defense responses are activated during the night in response to aphid infestation is unknown and warrants further investigation. Interestingly, our data show the time of day did not impact the total time spent in phloem ingestion, the second subpattern observed within the SEP. Two parameters that reflect phloem suitability and persistence in phloem ingestion, percentage of aphids showing sustained phloem ingestion and the potential E2 index (van Helden & Tjallingii, 1993) were both higher during night feeding. Taken together, it is plausible that the increased salivation by aphids observed during the night serves to increase the suitability of the phloem for persistent feeding. A similar response has been observed in Russian wheat aphid feeding behavior monitored using the EPG technique on five different wheat genotypes. The aphids spent significantly longer times in the sieve element phase during the night (Ni & Quisenberry, 1997). However, the authors failed to breakdown the SEP into salivation and ingestion phases. Therefore, it is unclear whether the aphids spent longer times in salivation or phloem ingestion.

Dehydration in aphids causes hyperosmotic stress and could potentially have long term effects of the health and performance of the aphid. In our study, we found that the body water contents of the aphids were higher after night-time feeding (76.2% $\pm$ 2.5%) as compared to day-time feeding (72.4% $\pm$ 2.1%). Our experimental set-up did not allow for determining the long-term effect of reduced body water content on aphid health and fecundity. However, other studies have highlighted the long-term impact of reduced body moisture in aphids. Nalam *et al*. (2012) show that in the event that aphids are unable to consume xylem sap, their body water content reduces and this results in reduced fecundity. Daniels *et al*. (2009) show that treatment with a sublethal dose of the neonicotinoid, thiamethoxan, resulted in dehydration (reduction in body water content from 75.6% $\pm$ 0.18% to 74.5% $\pm$ 0.23%) of the aphid and reduced performance including reduction in body size and fecundity. Guo *et al*. (2016) show that in plants under drought stress the relative body water content of aphids is reduced compared to on well-watered plants and this is associated with a reduced fecundity of the drought stressed plants.

We hypothesized that night-time feeding by aphids takes advantage of a lower sugar content in the phloem sap to compensate for osmotic stress incurred during day-time feeding. And indeed, we observed that at the end of night-feeding, *R. padi* are significantly more hydrated as compared to aphids sampled at the end of the day. This observation is in agreement with previous findings that phloem sap composition in wheat plants undergoes diurnal variation in sugar and amino acid content (Hayashi & Chino, 1986; Caputo & Barneix, 1999; Gattolin *et al*., 2008; Taylor *et al*., 2012). Studies in plant species other than wheat such as tree tobacco (Hocking, 1980), muskmelon (Mitchell *et al*., 1992), apple (Klages *et al*., 2001), castor bean and tansy (Kallarackal *et al*., 2012) have shown that diurnal fluxes occur in plant phloem sap especially with respect to sucrose with concentrations and/or flux being lower during the night. Although phloem sap content of sucrose in wheat plants was not measured in our study, we have several lines of evidence that support the findings that diurnal variation with respect to sucrose concentrations does exist in wheat phloem. First, aphids are significantly more hydrated after night feeding. Second, the genes involved in osmoregulation, gut *sucrase 1* and *aquaporin 1*, show a diurnal pattern of expression with lower levels of the transcripts observed during the night. Third, a significantly smaller proportion of aphids feed from the xylem during the night-time suggesting that xylem consumption during the night is not essential to achieve osmoregulation. Fourth, aphids feeding during the night spent longer times in salivation suggesting that aphids are secreting effectors to either suppress plant defense or induce sugar transport in the phloem. The increased salivation during the night may also contribute to greater phloem acceptability as indicated by the longer time spent on sustained ingestion during the night. Although honeydew production was not measured in our study, our results are in agreement with previous reports that honeydew production by aphids is reduced during the night owing to the more dilute sap in the plant at night (Cull & Emden, 1977; Taylor *et al*., 2012). These data highlight

the need for improved understanding of feeding behavior of insects in relation to diurnal physiology of its host plant.

Overall, this study demonstrates that in spite of the presence of a diurnal rhythm in a core circadian clock gene, aphids exhibit similar feeding behaviors during the day and night. We suggest that aphids take advantage of the diurnal variation in phloem sap content for osmoregulation. Aphids sampled at the end of a night of feeding on the host plant, were more hydrated as compared to aphids sampled at the end of a day. In conclusion, we show that in addition to the previously known mechanisms of osmoregulation (Fig. 5), night-time feeding is a general behavioral response displayed by aphids that takes advantage of the diurnal variation in phloem sap content.

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Disclosure

The authors declare no competing financial interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Gene specific primers used in the study.
Table S2. Results of COSINOR analysis.