A phloem-sap feeder mixes phloem and xylem sap to regulate osmotic potential

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Phloem-sap feeders (Hemiptera) occasionally consume the dilute sap of xylem, a behaviour that has previously been associated with replenishing water balance following dehydration. However, a recent study reported that non-dehydrated aphids ingested xylem sap. Here, we tested the hypothesis that the consumption of xylem sap, which has a low osmolality, is a general response to osmotic stresses other than dehydration. Alate aphids were subjected to different treatments and subsequently transferred onto a plant, where electrical penetration graph (EPG) was used to estimate durations of passive phloem sap consumption and active sucking of xylem sap. The proportion of time aphids fed on xylem sap (i.e., time spent feeding on xylem sap/total time spent feeding on phloem plus xylem sap) was used as a proxy of the solute concentration of the uptake. The proportion of time alate aphids fed on xylem sap increased: (1) with the time spent imbibing an artificial diet containing a solution of sucrose, which is highly concentrated in phloem sap and is mainly responsible for the high osmotic potential of phloem sap; (2) with the osmotic potential of the artificial diet, when osmotic potential excess was not related to sucrose concentration; and (3) when aphids were deprived of primary symbionts, a condition previously shown to lead to a higher haemolymph osmotic potential. All our results converge to support the hypothesis that xylem sap consumption contributes to the regulation of the osmotic potential in phloem-sap feeders.

1. Introduction

Phloem sap is a nutrient-rich food source, and the exclusive source of nutrients to most Hemipterans, including aphids, whiteflies, mealybugs, scale insects and psyllids from the suborder Sternorrhyncha, most of the plant-hoppers and many leaf-hoppers from the suborder Auchenorrhyncha, and lygaeids, pentatomids and coreids from the suborder Heteroptera (Dolling, 1991). Many of these phloem-sap feeders represent important agricultural pests (Resh and Cardé, 2003). However, to exploit phloem sap these insects must overcome the high osmotic potential (solute concentration per kg of solvent) of phloem sap, caused primarily by the high sucrose concentration (Douglas, 2006). The osmotic potential gradient between the ingested sap in the stomach and haemolymph (i.e., insect blood) could lead to the transfer of water from the body fluids to the gut, resulting in osmotic stress in the haemolymph. Osmoregulation in animals can be achieved by excreting the excess compounds or by gaining water from food or drinking (Albers and Bradley, 2004). However, Malpighian tubules, which secrete the excess compounds in many insects (Albers and Bradley, 2004), are absent from phloem-sap feeder species (Dixon, 1998), and the characteristics of the hemipteran mouthparts (stylets) prevent phloem-feeders from imbibing fluids outside of plant vascular cells, epidermal and mesophyll cells (Dixon, 1998), and artificial diets (Mittler and Dadd, 1963).

Previous studies on aphids established that the osmotic potential of the gut contents can be regulated through two sucrose-related physiological processes. After cleavage of sucrose into its two moieties (fructose and glucose), the fructose is assimilated by the organism (Febvay et al., 1999; Ashford et al., 2000), while several glucose molecules are polymerised into oligosaccharides (Wilkinson et al., 1997; Ashford et al., 2000). Both phenomena reduce the concentration of sucrose in the stomach.Sucrose assimilation rate is partly modulated by the obligate microbial symbiont of aphids, Buchnera aphidicola (Febvay et al., 1999; Wilkinson and Ishikawa, 1999), which every aphid species harbour. In the pea aphid, the elimination of symbionts through antibiotic treatment leads to a higher haemolymph osmotic potential (Wilkinson et al., 1997). Synthesised oligosaccharides are then excreted through honeydew, which is iso-osmotic with haemolymph for insects reared on plants (Wilkinson et al., 1997). To complement the sucrose-related processes, water transfer from the hind gut, where the osmotic potential has already been reduced, to the stomach can...
occur because of the close apposition of the two organs. Water transfer from the hind gut reduces the osmotic potential of stomach contents (Douglas, 2003; Shakesby et al., 2009). These previously described osmoregulation processes are summarized in Fig. 1.

In contrast to phloem sap, which is under positive hydrostatic potential (Kennedy and Mittler, 1953), xylem sap has to be actively sucked resulting in an energetic cost associated with its consumption (Malone et al., 1999). However, aphids (Tjallingii, 1995), whiteflies (Lei et al., 2001), psyllids (Bonani et al., 2010), plant-hoppers (Khan and Saxena, 1984) and leaf-hoppers (Stafford and Walker, 2009) occasionally consume xylem sap. Xylem sap contains salts and some amino acids, but at a lower osmolality than phloem sap (measured during the day), although it is sufficient to sustain xylem-sap feeding insects (Andersen et al., 1989). Xylem sap consumption was first noticed in dehydrated alate aphids (Spiller et al., 1990), and thus was associated with replenishing water following the dehydration period prior to flight (Powell and Hardie, 2002). Dehydration during the teneral period of alate aphids results in a loss of weight (MacKay and Downer, 1979), which is thought to be adaptive as it should facilitate subsequent flight by decreasing the wing loading (Dixon and Kindlmann, 1999). However, a recent study showed that both well hydrated alate and apterae aphids ingest xylem sap when their fecundity decreases (Pompon et al., 2010), thus suggesting that there is another reason, in addition to dehydration, for mixing phloem and xylem sap.

Here, we report results testing three predictions emanating from the hypothesis that the consumption of a dilute diet (xylem sap), thereby potentially decreasing osmotic potential (Cull and Van Emden, 1977), is a behavioural response to reduce osmotic stress (hypothesis illustrated in Fig. 1). Using a common aphid pest of potato (Macrosiphum euphorbiae), we show that the proportion of xylem sap fed on xylem sap (i.e., time spent feeding on xylem sap/total time spent feeding on phloem and xylem sap) increases: (i) with the duration of feeding on a sucrose artificial diet; (ii) with the osmotic potential of the diet, when osmotic potential increases independently of sucrose concentration; and (iii) to compensate for an increase in haemolymph osmotic potential resulting from symbiont depletion as observed for the pea aphid (Wilkinson et al., 1997).

2. Materials and methods

2.1. Aphids and plants

Potato plant (Solanum tuberosum var. Shepody) growing conditions and the source and rearing of M. euphorbiae aphids (Hemiptera: Aphididae), were as previously described (Pelletier et al., 2010), and only allowed aphids to reproduce parthenogenetically. We standardized the age of alate adult aphids as previously described (Pompon et al., 2010). In brief, alate adults were removed from the ceiling of the rearing cage (1 m high, 50 cm deep and wide, and all sides and ceiling screened) that was sufficiently large to allow alate aphids to engage in flight. After 14 h, alate aphids were collected from the ceiling and walls of the cage, and used in experiments. As alate aphids fly from the plant less than 24 h after molting and do not take off once settled on a suitable plant (Robert, 1988), collected aphids were approximately 1-day-old. All aphid manipulations were carried out with a soft-bristled paint brush.

2.2. Feeding behaviour assessment

Electrical penetration graph (EPG) provides a powerful tool to study aphid feeding behaviour by differentiating passive phloem sap consumption from active sucking of xylem sap, as well as other probing and feeding behaviours on both plant and artificial diet (Tjallingii, 1995). EPG was conducted in an electrically grounded Faraday cage to shield the setup from external electrical noise. All experiments were carried out in a laboratory environment under constant light at a temperature of 20 °C. A fine gold wire (2–3 cm long and 12 μm in diameter) was glued with water-based silver conductive paint to the dorsum of a 1-day-old alate aphid immobilized with a vacuum device (van Helden and Tjallingii, 2000). The substrate electrode consisted of a copper stalk (10 cm) inserted into the soil of the pot of the studied plant, whereas on artificial diet, a metal mesh running in the liquid solution of the artificial diet was used. Both the substrate electrode and the aphid wire were connected to a Giga 8™ amplifier with 107 Ω input resistance (EPG-systems, Wageningen, The Netherlands). One wired aphid was deposited on one young leaf (2nd to 3rd level from the apex) of one plant or on one artificial diet sachet (see below), and EPG was recorded for 4 and 2 h, respectively, using Scope software (Data Translation, Marlboro, USA). Plants and artificial diets were only used once. When the aphid inserts its stylect, fluctuations in the electrical resistance and electromotive forces in the aphid–plant combination result in variations of the electrical potential, which provide typical waveforms indicative of behavioural activities (Tjallingii, 1990). Transfers from artificial diet to a host plant were carried out in less than 5 min to prevent any dehydration modifying xylem sap consumption behaviour (Powell and Hardie, 2002).

To calculate the proportion of time aphids spent ingesting xylem sap, hereafter referred to as the proportion of xylem sap ingested, the duration of xylem sap (G waveform) consumption was divided by the durations of phloem sap (E2 waveform) plus xylem sap consumption. The proportion of xylem sap ingested provided a standardized value as duration of sap intake (phloem plus xylem saps) may vary among aphids within a given observed time period (Prado and Tjallingii, 1997). The proportion of xylem sap ingested was arcsine-transformed before analysis, and normality of

![Fig. 1](image-url) Functional scheme of osmoregulation in phloem-sap feeders. The symbols + and – represent positive and negative relationships, respectively. The straight line separates events occurring in the aphid body from others involving the exterior environment, while the broken line represents the permeable membrane between the gut and haemolymph. Osmoregulation as previously described (Douglas, 2003, 2006): the osmotic pressure in stomach and haemolymph are related; when insects ingest phloem sap, which has a high concentration of sucrose, the osmotic potential in the stomach increases; osmotic potential in the stomach is reduced by sucrose assimilation through sucrose metabolism and the synthesis of oligosaccharides; sucrose metabolism is positively influenced by symbiont abundance and nymph production, which is itself positively related to symbiont abundance; oligosaccharides are excrated through honeydew, and their synthesis rate increases with sucrose concentration in the stomach; water transfer from the hind gut, where osmotic potential has already been reduced, to the stomach can occur to dilute the osmotic potential in the stomach. Our hypothesis is that osmotic stress stimulates xylem sap consumption, which results in a reduction of the osmotic potential of the stomach content.
the distributions was checked using the Kolmogorov–Smirnov test. When aphids fed on artificial diets that were not under positive hydrostatic pressure, waveform G (characteristic of active uptake on plant) (Spiller et al., 1990) was observed and used to calculate the time spent ingesting the artificial diet. Durations of the E2 and G waveforms are indicative of the quantities of phloem sap ingested on plants (Prado and Tjallingii, 1997) and artificial diet (Tjallingii, 1978), respectively. No direct assessment of the relationship between G waveform duration and the amount of xylem sap ingested in planta has been achieved so far. Nonetheless, the duration of the G waveform is commonly used to indicate the quantity of xylem sap ingested (Powell and Hardie, 2002; Daniels et al., 2009).

2.3. Artificial diet

Two hundred microlitre of a liquid diet were deposited between two stretched sheets of Parafilm® over a yellow (stimulating feeding behaviour (Pelletier, 1990)) cap (9 cm diameter and 1 cm deep), with a thin metal mesh running between the two Parafilm® sheets. We used two different diets: 0.4 M sucrose diluted in distilled water, and the same sucrose solution to which was added 0.01 M of inulin (Sigma–Aldrich, Oakville, ON, Canada). Inulin was diluted in hot distilled water as the concentration used corresponds to its maximum water solubility, and cooled before use. Osmotic potentials of the artificial diets were measured with a vapour pressure osmometer (Model 5500, Wescor, Inc., Logan, UT, USA).

2.4. Symbiont community structure

To determine the bacterial symbiont community of the aphid colony, PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE), using the same universal primers targeting the variable V3 region of the bacterial 16S rRNA gene (Muyzer et al., 1993), was performed as detailed previously (Muyzer et al., 1993). Total DNA was extracted for 10 replicates of one-day-old alate aphid with the QIAamp tissue kit (Qiagen, Valencia, CA, USA) following manufacturer’s instructions. PCR reactions were performed using high-fidelity Pfx50 DNA polymerase (Invitrogen, Burlington, ON, Canada). DGGE bands were visualised by Sybr-Green® (Invitrogen, Burlington, ON, Canada) staining, excised, and incubated overnight in 100 µl distilled water. Sequence purity of the excised band was checked again through PCR-DGGE and then sequenced (Genome Québec, Québec, QC, Canada). Sequences were aligned with ClustalW and blasted in NCBI nucleotide database.

2.5. Deprivation and quantification of the density of B. aphidica symbiont

One-day-old nymphs were reared on antibiotic-containing (100 µg/ml rifampycin) (Fisher, Ottawa, ON, Canada) and control (without antibiotic) full (amino-acids, vitamins, and sucrose as described previously (Febvay et al., 1988)) artificial diets for 4 days (20 individuals per artificial diet), and kept individually on a potato plant leaf after the treatment until adult moult. Alates were assessed the day of their adult moult.

Ten aphids per treatment (antibiotic-treated and control) were individually weighed (MTS, Mettler, Mississauga, ON, Canada) before extracting total DNA with a QIAamp tissue kit (Qiagen, Valencia, CA, USA) following manufacturer’s instructions. Absolute quantification of the 16S rRNA gene of B. aphidica was achieved in triplicate for each sample using the stepOnePlus™ system (Applied Biosystem, Foster City, CA, USA) in 25 µl reaction mix containing 1X powersYBR® green PCR master-mix (Applied Biosystem, Foster City, CA, USA), 0.3 µM of each specific primer Buch16S1F and Buch16S1R (Tsuchida et al., 2002), and 1 µl of sample. The PCR program included 10 min at 95 °C and 50 cycles (95 °C for 30 s, 60 °C for 15 s, 72 °C for 30 s) and fluorescence was measured at 78 °C for 30 s to remove signals that may have been obtained from primer dimers. The Buchnera density (per mg of aphid and per individual aphid) of each sample was calculated by averaging the three replicate values. External standard curves were generated from PCR products purified with the QiAquick PCR purification kit (Qiagen, Valencia, CA, USA). The size of the purified PCR product (432 bp) was confirmed by sequencing (Genome Québec, Québec, QC, Canada). Copy number of the fragment was calculated from the length (base pairs) and the concentration of the purified PCR product, obtained by using the Nanodrop spectrophotometer (Termo Scientific, Wilmington, DE, USA). One-tenth serial dilutions of PCR products from a sub-sample of the aphid colony covered a range from 10^6 to 10^9 copies/ml. Standard curves were run in triplicate on each 96-well plate used for real-time PCR. A single copy of the 16S rRNA gene is present in the B. aphidica genome (Shigenobu et al., 2000).

3. Results

3.1. Influence of the time spent ingesting a sucrose solution on the subsequent proportion of xylem sap ingested

To test whether the duration of sucrose ingestion positively influences the proportion of xylem sap ingested, we monitored the feeding behaviour of eighteen aphids on artificial diets containing a 0.4 M sucrose solution (in distilled water), and after they were transferred onto potato plants. The time spent ingesting the sucrose diet, recorded using EPG, varied among individual aphids (Fig. 2), but was positively related to the subsequent proportion of xylem sap ingested on the plant (Y = 0.0004 X + 0.225, R^2 = 0.46, P = 0.002). We verified the normal distribution of error terms using the Shapiro–WilK normality test.

All insects that for more 1100 s on the artificial diet only consumed xylem sap (Fig. 2). This saturating effect is likely due to the experimental settings. Had we left the aphids for a longer period on the plant, it would have had the time to switch to phloem sap consumption after balancing its osmotic pressure by imbining xylem sap. Accordingly, we observed that aphids always started by ingesting xylem sap and most of the time did not go back to xylem sap ingestion during the assessed duration (data not shown).

Fig. 2. Relationship between the time aphids (n = 18) spent ingesting the sucrose solution artificial diet within a 2 h exposure period and the subsequent proportion of time spent ingesting xylem sap on plant within a 4 h exposure period. Classical linear regression visualised by the dotted line was significant (Y = 0.0004 X + 0.225, R^2 = 0.46, P = 0.002).
3.2. Influence of diet osmotic potential on the subsequent proportion of xylem sap ingested

To test whether xylem sap consumption is a response to an increase in osmotic potential of the diet but not to sucrose ingestion per se, we monitored the feeding behaviour of 28 aphids on two artificial diets (14 on each) containing the same concentration of sucrose (corresponding to 400 mOsmol L⁻¹) but different osmotic potentials, and after their transfer onto potato plants. The excess of osmotic potential was generated by adding inulin (corresponding to an additional 10 mOsmol L⁻¹). As inulin cannot be metabolized by aphids (Wright et al., 1985), the osmotic potential excess cannot be regulated using physiological processes.

Aphids fed for similar periods of time on the two diets (1049 s on the sucrose diet and 1119 s on the sucrose diet with inulin; t-test: df = 26; P = 0.82), which suggests that the augmented osmotic potential and the addition of inulin did not interfere with feeding on the artificial diets. Sucrose concentration influences artificial diet uptake (Douglas et al., 2006) and was equal in both diets. When aphids were subsequently transferred onto a plant, the proportion of xylem sap, normalized per ingestion time of the artificial diet, was higher for aphids that fed on the diet with the higher osmotic potential (ANOVA: F(1, 26) = 4.82; P = 0.037; Fig. 3).

3.3. Influence of symbiont depletion on the subsequent proportion of xylem sap ingested

To test whether aposymbiotic (antibiotic-treated) insects compensate for an increased haemolymph osmotic potential by consuming a higher proportion of xylem sap, we compared the feeding behaviour on plants of 10 aphids treated with antibiotic to 10 non-treated aphids. Efficiency of the antibiotic treatment was assessed by quantifying the abundance of the symbiont detected in the aphid colony. The presence of one unique and identical band on PCR-DGGE gels (Fig. 4) indicated that there was only one symbiont species in the aphid colony. The DNA sequence of the band had 100% homology with the obligatory symbiont B. aphidicola from the aphid Myzus persicae (Hemiptera: Aphidoidea) (M63249.1). The antibiotic treatment had a significant effect on B. aphidicola density per mg (ANOVA: F(1, 17) = 14.78; P < 0.001; one sample was below the detection threshold of the qPCR assay) and per individual aphid (F(1, 17) = 15.42; P < 0.001) as measured with quantitative PCR (Fig. 5A). Notably, the antibiotic treatment reduced but did not totally eliminate symbionts: B. aphidicola mean (±s.e.m.) density per mg of aphid was \(1.44 \times 10^4\) (±7.26 × 10³) after antibiotic treatment (Fig. 5A). When released on plants, antibiotic-treated aphids spent a higher proportion of time ingesting xylem sap (ANOVA: F(1, 18) = 17.82; P < 0.001; Fig. 5B).

4. Discussion

The results of the three experiments supported the hypothesis that aphids experiencing an osmotic stress mix phloem and xylem...
sap to regulate osmotic potential. In the experimental settings, we assumed that the duration of xylem sap consumption would serve as a proxy for the quantity of xylem sap the aphid would imbibe on the plant. Previous studies reported a direct link between the duration of phloem sap consumption and the quantity ingested (Prado and Tjallingii, 1997), but no one before this study has established the same relationship for xylem sap. Nevertheless, a positive correlation between ingestion time on the artificial diet (indicated by the same waveform as for xylem sap ingestion on a plant) and the actual quantity imbibed (Tjallingii, 1978) has previously been cited to justify the use of the duration of xylem sap ingestion as an estimate of the quantity of xylem sap ingested (Powell and Hardie, 2002; Daniels et al., 2009).

First, we observed that the proportion of xylem sap ingested on plants was positively influenced by the amount of time an aphid had previously spent feeding on a sucrose solution diet (Fig. 2). Haemolymph osmotic pressure of aphids increases with the sucrose concentration of the artificial diet (Douglas et al., 2006). The quantity of sucrose ingested from the artificial diet likely augmented the gut osmotic potential, which could then be lowered by imbibing xylem sap. Second, the proportion of xylem sap ingested on plants increased with the osmotic potential of the diet previously imbied (Fig. 3). The osmotic potential excess was created by adding a compound not metabolized by the aphid and by maintaining a constant sucrose concentration. Hence, aphids were prevented from implementing sucrose-related physiological processes (sucrose assimilation and polymerisation) to regulate the osmotic potential excess. Our results also indicated that it is the osmolality of the ingested diet and not the sucrose concentration that stimulates xylem sap consumption. Similarly, a reduction in the abundance of the obligatory symbiont resulted in an increase in the proportion of xylem sap ingested (Fig. 5). Previous studies demonstrated that symbiont depletion triggers an increase in haemolymph osmotic potential in the pea aphid (Wilkinson and Ishikawa, 1999), but had no influence on non-phloem feeding behaviour (Wilkinson and Douglas, 1995). However, precise measurement of the duration of xylem sap consumption and the proportion of xylem sap ingested were not included in the previous study (Wilkinson and Douglas, 1995). The normalization of xylem sap consumption over total sap intake may be required to gain information about the concentration of ingested solute in the gut. Nonetheless, a direct measurement of haemolymph osmotic pressure after the different treatments would strengthen our findings.

Because dehydration causes hyperosmotic stress, our conclusion does not contradict previous reports that aphids consume xylem sap to rehydrate (Spiller et al., 1990; Ramírez and Niemeyer, 2000; Powell and Hardie, 2002; Daniels et al., 2009), but rather extends the former hypothesis by demonstrating that xylem sap consumption is a general response to osmotic stress, as previously hypothesized by Pompon et al. (2010).

High sucrose concentration in phloem sap exerts an osmotic stress on phloem-sap feeders (Douglas, 2006). To overcome this physiological barrier, phloem-sap feeding species have evolved osmoregulation processes (illustrated in Fig. 1) that function synergistically. For instance, oligosaccharide polymerisation can be enhanced to compensate for a reduction in sucrose assimilation (Wilkinson et al., 1997; Wilkinson and Ishikawa, 1999). However, by themselves, physiological mechanisms are not sufficient to permit aphids to feed on artificial diets with the high sucrose concentrations (Douglas et al., 2006) measured in field plants (Fisher, 2000). Biological performance on artificial diets has been observed to increase when aphids are transferred to a low sucrose diet for a few hours each day (Douglas and Van Emden, 2007). We speculate that phloem-sap feeders can consume xylem sap to alleviate the osmoregulation limit of the physiological processes, thus enabling them to feed on plant sap with high sucrose concentrations. This conclusion can now explain the inverse relationship between the proportion of xylem sap ingested and fecundity in both alate and apterous aphids, observed earlier (Pompon et al., 2010). Fecundity is the major sink of sucrose metabolism (Febvay et al., 1999; Ashford et al., 2000) and its decrease in old aphids (Pompon et al., 2010) likely results in a reduction in sucrose assimilation, which in turn decreases physiological osmoregulation capacity. In such a case, aphids would consume xylem sap to compensate for the limited physiological osmoregulation capacity.

In conclusion, we validated three independent predictions supporting the hypothesis that xylem sap consumption is a general response to osmotic stress. Aphids exploit xylem sap to regulate the osmotic potential of their diet, when osmotic potential is caused by sucrose or a non-metabolized compound. Increased haemolymph osmotic potential resulting from impairment of the physiological processes participating in osmoregulation stimulated xylem sap ingestion. Our results suggested a behavioural mechanism of osmoregulation in Hemiptera.

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