Impact of environmental stress on aphid clonal resistance to parasitoids: Role of Hamiltonella defensa bacterial symbiosis in association with a new facultative symbiont of the pea aphid

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ABSTRACT

Resistance to endoparasitoids in aphids involves complex interactions between insect and microbial players. It is now generally accepted that the facultative bacterial symbiont Hamiltonella defensa of the pea aphid Acyrthosiphon pisum is implicated in its resistance to the parasitoid Aphidius ervi. It has also been shown that heat negatively affects pea aphid resistance, suggesting the thermosensitivity of its defensive symbiosis. Here we examined the effects of heat and UV-B on the resistance of A. pisum to A. ervi and we relate its stability under heat stress to different facultative bacterial symbionts hosted by the aphid. For six A. pisum clones harboring four different facultative symbiont associations, the impact of heat and UV-B was measured on their ability to resist A. ervi parasitism under controlled conditions. The results revealed that temperature strongly affected resistance, while UV-B did not. As previously shown, highly resistant A. pisum clones singly infected with H. defensa became more susceptible to parasitism after exposure to heat. Interestingly, clones that were superinfected with H. defensa in association with a newly discovered facultative symbiont, referred to as PAXS (pea aphid X-type symbiont), not only remained highly resistant under heat stress, but also expressed previously unknown, very precocious resistance to A. ervi compared to clones with H. defensa alone. The prevalence of dual symbiosis involving PAXS and H. defensa in local aphid populations suggests its importance in protecting aphid immunity to parasitoids under abiotic stress.

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1. Introduction

Under natural conditions, aphids and their bacterial symbionts as well as their associated parasitoids are exposed to a variety of environmental stresses. Even though each organism in such associations may be individually adapted to fluctuating temperature and solar radiation regimes, the relative complexity of their interactions may render their associations intrinsically more vulnerable to any important deviations from their respective adaptive range (Thomas and Blanford, 2003). In the context of climate change that is predicted to cause a global increase in temperatures, or because of the thinning of the ozone layer leading to higher UV-B radiation, it is important to better understand the dynamics of complex associations such as aphids and how they may respond to long term change (Caldwell et al., 2007; Hance et al., 2007).

Several studies have already confirmed the negative consequences of heat stress on the pea aphid Acyrthosiphon pisum Harris (Hemiptera: Aphididae), especially on viviparous reproduction, ranging from a reduction in fecundity to complete sterilization (Ohtaka and Ishikawa, 1991). Such a marked impact is largely due to the elimination by high temperatures of Buchnera aphidicola, the obligatory nutritional bacterial symbiont of aphids (Douglas, 1998). Other studies have demonstrated that A. pisum facultative bacterial symbionts can also play a role in situations of heat stress, suggesting that clones with specific facultative symbiotic bacteria could sustain high fecundity and other key fitness traits under hot conditions (Chen et al., 2000; Montllor et al., 2002; Russell and Moran, 2006). Some facultative symbionts, like Serratia symbiotica, could protect aphids against high temperature, in saving B. aphidicola by maintaining a high number of bacteriocytes (or mycetocytes), the aphid cells in which most of the bacterial symbionts reside (Montllor et al., 2002). Direct UV-B radiation is another kind of abiotic stress that alters cellular integrity and damages DNA in most living organisms, and which is also believed to affect aphid fitness. Recent work within our group on the potato aphid Macrosiphum euphorbiae did not reveal any direct effects of
UV-B on reproduction (Nguyen et al., 2009), yet this work was not designed to address the potential role of facultative bacterial symbionts in protecting aphids. One objective of the present study was to examine the impact of heat and UV-B radiation on pea aphid fecundity, and whether the impact may vary depending of the types of facultative symbionts harbored by the host.

Another aspect of aphid biology that can be disrupted following exposure to stressful conditions is the immune response to parasitism. While some Diptera and Lepidoptera rely on complex cellular responses like encapsulation to escape parasitism (Lavine and Strand, 2002; Schmid-Hempel, 2005), encapsulation seems to be absent in aphids (Henter and Via, 1995; Ferrari et al., 2001; Bensadia et al., 2006). In A. pism, it has however been shown convincingly that facultative symbiotic bacteria, particularly Hamiltonella defensa, possibly in association with the phage APSE (Moran et al., 2005a; Degnan and Moran, 2008a,b), can determine resistance to parasitoids (Oliver et al., 2003, 2005; Ferrari et al., 2004), even though the implicated mechanisms are still unknown. Subsequently, Bensadia et al. (2006) demonstrated the negative impact of high temperatures on the ability of A. pism clones to resist establishment of the parasitoid Aphidius ervi Haliday (Hymenoptera: Braconidae), as observed under normal conditions. Individuals harboring H. defensa and fully resistant at moderate temperatures (20°C) became much more susceptible to parasitism at higher temperatures (25°C and 30°C), leading to the hypothesis that defensive bacterial symbionts are differentially affected by exposure to heat, which would explain the loss of clonal resistance to A. ervi.

The impact of UV-B radiation on aphid resistance to parasitoids apparently has not been studied yet. However, if the facultative symbionts that are responsible for resistance to parasitoids are susceptible to heat stress, they may also be sensitive to UV-B stress as these two kinds of abiotic stress are often closely associated in nature for insects living on plants. This is especially true in aphids such as A. pism, which are very small insects and usually poorly melanized, and hence potentially less protected against radiative stress. If resistance to parasitoids is dependent on the presence of facultative symbiotic bacteria, these may also be affected by UV-B radiation, which would lead to increased levels of parasitism.

The main objective of this work was to measure the impact of heat and UV-B radiation on A. pism ability to resist A. ervi parasitism, using several pea aphid clones harboring different facultative bacterial symbionts. This was achieved by exposing two consecutive generations of aphids (G1 and G2) to a combination of stressful, and non-stressful abiotic conditions. We measured the level of resistance of G2 aphids to A. ervi, and the fecundity of the G1 aphids, which served here as a control to assess the magnitude of our experimental stressful conditions.

2. Methods

2.1. Insects and bacterial symbionts

Several clonal lineages of A. pism are maintained in our laboratory. The clonal colonies originated from individuals collected on red clover (Trifolium pratense) and alfalfa (Medicago sativa) at two distant geographical locations, Québec (QC, Canada) and Madison (WI, USA). Each clone was reared individually on shoots of young broad bean plants (Vicia faba) contained in small cages at 20 ± 1°C, 65 ± 5% RH, under a 16L:8D photoperiod. For the experiments, aphids were kept in small Petri dishes (50 mm × 15 mm, Fisher Scientific Company, Ottawa, ON, Canada) containing a broad bean leaf disc (3 cm diameter) inserted in a 0.8% agar medium (Sigma, Oakville, ON, Canada). Petri dishes were covered with a lid to confine the aphids and ventilated through two lateral openings (20 mm × 5 mm) made of muslin to avoid water vapor condensation.

Experimental A. pism clones were selected according to their level of resistance to the parasitoid A. ervi and their facultative bacterial symbiont associations. Susceptibility assays were conducted separately for each clone using one mated female parasitoid that was allowed to perform a single attack on 10 L2 individuals of the same clone that were kept on broad bean shoots for 15 days. Host aphid mummification and parasitoid emergence rates were used as indicators of the susceptibility of the clones to A. ervi parasitism. The clones were characterized for three major facultative symbionts of A. pism (S. symbiotica, Regiella insecticola, H. defensa; Moran et al., 2005b) by molecular techniques involving diagnostic PCR, restriction digestion and sequencing using bacterial 16S rDNA as described in Sandström et al. (2001). In all, six experimental clones were selected (Table 1) for their widely variable levels of resistance to A. ervi (four highly resistant, two highly susceptible) and their possession of four different facultative symbiont associations: S. symbiotica, R. insecticola, H. defensa, and H. defensa in association with PAXS, a newly characterized symbiont of A. pism (see Section 3 below).

The A. ervi wasps came from a laboratory colony established with mummies of A. pism collected on alfalfa in Madison (WI, USA). They were reared on a non-resistant A. pism clone maintained on broad bean at 20 ± 1°C, 65 ± 5% RH, under a 16L:8D photoperiod. Individuals used for parasitism assays were previously isolated at the mummy stage and mated under direct observation after emergence. The mated females used in the experiments were all aged between 48 h and 120 h post-emergence.

2.2. Experimental setup

This experiment involved two main factors, temperature and UV-B radiation, each with two different levels (control and stress) to form a classic 2 × 2 full factorial design with four treatments: T°C UV−, T°C UV+, T° UV−, and T° UV+. 2.2.1. Temperature

The two temperature treatments were a control constant temperature regime (20 ± 0.5°C) and a stressful fluctuating temperature regime (4 h at 35 ± 0.5°C + 20 h at 17 ± 0.5°C). Each regime was programmed separately in a Conviron plant growth chamber (Controlled Environments Limited, Winnipeg, MB, Canada) with a relative humidity of 65 ± 5% and a 16L:8D photoperiod. Environmental parameters (temperature, relative humidity and photoperiod) were continuously recorded during the experiments with HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA). For each temperature regime, the average daily temperature was 20 ± 1°C, so as to ensure a similar development rate for aphids in both regimes by exposing them to the same amount of heat per 24 h period (Lamb, 1992). In the stressful regime, the 4 h exposure to high temperature corresponded to the mid-day period (11 a.m. to 3 p.m.), when temperature is normally at its highest under natural conditions.

2.2.2. UV-B radiation

Each of the two growth chambers with controlled temperature regimes was subdivided using a light proof vertical panel to create the two UV-B radiation regimes (with or without UV-B stress). The UV source was made of two fluorescent tubes (30 W, 90 cm, Exo Terra Repti Glo 8.0, 33% UV-A and 8% UV-B, Roif C. Hagen, Montréal, QC, Canada) positioned 35 cm above the Petri dishes with leaf discs containing the exposed aphids. UV-B flux was measured with a UVP UV Radiometer (UVP, Upland, CA, USA). The average irradiance measured directly above the Petri dish was 0.62 W m−2. Because the Petri dish lids reduced the effective irradiance at the level of aphids by about 40% to 0.37 W m−2, the
2.3. Experimental procedures

The stress experiment involved observations on two successive generations of aphids (G1 and G2) exposed to experimental conditions, which allowed to consider the telescoping of generations in viviparous aphids (Dixon, 1998, pp. 83–84). Complete replications of the experiment (n = 10) were performed consecutively.

2.3.1. First-generation aphids (G1)

For each treatment, five young L1 aphids (0–24 h) of each clone were transferred in a Petri dish containing a broad bean leaf disc. The Petri dishes were then transferred to their assigned treatment, 1 h prior to the beginning of the heat stress. To prevent possible indirect stress on aphids caused by leaf disc exposure to the treatments, the Petri and leaf disc were renewed every 3 days or earlier, as necessary.

2.3.2. Second-generation aphids (G2)

Among the five L1 aphids initially exposed to the treatments, one apterous individual reaching the L4 stage was randomly selected to measure fecundity. The L4 aphid was transferred to a new Petri dish that was returned to its respective treatment and monitored for the beginning of reproduction. Viable progeny were subsequently counted daily, shortly before the heat stress period for the first 5 days of reproduction, while leaving the progeny with the adult. For each clone–treatment combination, three G2 aphids that reached the L2 stage were randomly selected to be kept aside for parasitism assays.

2.4. Parasitism assays

All aphids were tested for susceptibility to parasitism by direct observation of a parasitoid attack in a Petri dish, the A. ervi female being allowed to perform only one attack per host individual to limit the number of eggs laid to one. This procedure insured that the number of eggs and the amount of venom injected by the parasitoid at oviposition would be similar for all tested aphids in order to strictly control the variance of clonal resistance data.

To further control variance, each A. ervi female in the test was only allowed to attack three G2 aphids, all coming from the same clone–treatment combination. The experimental aphids were then transferred to a broad bean shoot inserted in a small cup of water covered with parafilm, which was placed in a small plastic cage with lateral muslin openings for ventilation. Cages were kept in a growth chamber programmed for standard conditions (20 ± 0.5 °C, 65 ± 5% RH, 16L:8D photoperiod). The aphids were monitored daily over a 4-day-period (96 h) corresponding to the time required by the A. ervi parasitoid to reach the first larval stage under standard conditions (Cloutier and Douglas, 2003; Nguyen et al., 2008). The presence or absence of an L1 parasitoid larva in each aphid was then observed by dissection under a binocular microscope, in 50 μl of phosphate buffered saline (PBS).

2.5. Statistical analysis

All statistical analyses were performed with SAS (v. 9.2, 2008). Fecundity and resistance data were analyzed using a randomized block ANOVA with a four-way treatment structure within each block, i.e. with the following fixed effects: temperature (n = 2), UV (n = 2), the symbiotic association (n = 4), and the clone (n = 6) which was nested in the symbiotic association effect. For both analyses, complete replications of the experiment were defined as blocks (n = 10), and treated as a random effect. The MIXED procedure of SAS was used to study the variance for aphid fecundity, where the data were counts. The GLIMMIX procedure was used for binary-type data obtained in parasitism assays, where observations were based on aphid dissection (presence or absence of an A. ervi L1 larva). All model assumptions were verified and the significance level was set at α = 5% for all analyses.

3. Results

3.1. Isolation of PAXS, a new facultative symbiont of A. pisum

Molecular characterization of experimental clones with H. defensa resulted in the isolation, in two highly resistant clones, of a new type of facultative symbiont that has not previously been described in A. pisum, and that will be referred to here as PAXS (or pea aphid X-type symbiont). Its characterization was made possible by restriction digest analyses on PCR-amplified eubacterial 16S rDNA digested with SalI. This restriction enzyme, known to cut the 16S rDNA of B. aphidicola but not the 16S rDNA of other facultative symbionts, produced in some clones digestion patterns differing from those of Sandström et al. (2001) (Fig. 1). The unknown digestion fragments obtained were isolated and sequenced, allowing us to design a specific forward primer, ‘PAXSF’ (5’-GAAGACATGCAAGAGTGTTGC-3’), that could be used in conjunction with the universal reverse primer ‘1507R’ of Sandström et al. (2001) for PAXS diagnostic purposes. A partial sequence of 1222 bp from the 16S rDNA gene of this symbiont was assembled for clone QCV1 (DNA Baser, v. 2.75, Heracle Software, Lilienthal, Germany) and deposited in GenBank (accession no. FJ821502). A BLASTN search allowed to confirm 98% sequence homology of PAXS with unidentified symbionts of the juniper aphid Cinara juniperi and the rosa aphid Maculolachnus submacula (Lamelas et al., 2008). When the PAXS sequence was compared against multiple 16S rDNA sequences and the available genome (CP001277) in GenBank of the symbiont H. defensa (taxid:138072), which co-infected aphid clones harboring PAXS, the maximum homology found was 92% for sequences of similar lengths.

Multiple alignments using ClustalX (v. 2.0.10, 2009) were made with the PAXS sequence and other 16S rDNA sequences of symbionts found in A. pisum and available in GenBank: B. aphidicola
(NC_002528), H. defensa (AF293616), R. insecticola (AF293618) and S. symbiotica (AF293617). Both sequences from unidentified symbionts of C. juniperi (EU348311) and M. submacula (EU348312) were also included, as well as an Escherichia coli sequence (NC_000913) which was used for reference. We confirmed a restriction site for Sall (5’-GTGCAC-3’) at position 1006 (E. coli numbering), which can account for the 16S rDNA fragment sizes obtained on gels (Fig. 1). That site is unique to PAXS and to unidentified symbionts of C. juniperi and M. submacula. When compared to other facultative symbionts of A. pisum, a 28-bp region specific to PAXS was also identified at E. coli positions 453–480 (Fig. 2). This region was however highly conserved in the C. juniperi and M. submacula symbionts, with the inclusion of a few nucleotide substitutions and deletions. Subsequent diagnostic PCR performed on other available clones in our laboratory allowed us to detect PAXS in two more H. defensa-infected clones collected on alfalfa in Québec (unpublished results).

3.2. Fecundity of G1 aphids

While most G1 aphids reproduced under all treatments, some aphids failed to do so under stress and others gave birth to aborted progenies whose legs and antennae did not deploy properly at birth. Typically, aborted progenies died within a few hours after birth and were not considered in the statistical analysis of fecundity.

Table 2
Statistical modeling of the impact of temperature and UV-B radiation on Acyrthosiphon pisum clones harboring different types of facultative bacterial symbionts on fecundity (A) and resistance to Aphidius ervi (B).

<table>
<thead>
<tr>
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<th>Den DF</th>
<th>F value</th>
<th>Pr &gt; F</th>
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</thead>
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<td></td>
</tr>
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<td>Block</td>
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<td>193</td>
<td>0.44</td>
<td>0.9093</td>
</tr>
<tr>
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<td>193</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>UV</td>
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<td>0.0043</td>
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<tr>
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<td>19.01</td>
<td>0.0001</td>
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<tr>
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<tr>
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<tr>
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(B) Resistance

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<tr>
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<tr>
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<td>0.90</td>
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<tr>
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<tr>
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<tr>
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<td>0.6490</td>
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<tr>
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<td>2</td>
<td>131</td>
<td>0.93</td>
<td>0.3338</td>
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</table>

Fecundity data were normally distributed, as confirmed by a Shapiro–Wilk test (P = 0.4432). The block effect (full replication of the experiment) was not significant (F3,193 = 0.44, P = 0.9093) and the analysis revealed a significant temperature × clone (symbiont) interaction (F2,193 = 6.72, P = 0.0015) (Table 2A). This indicates that clones harboring the same type of facultative bacterial symbionts did not always react similarly to temperature in terms of fecundity. Multiple comparisons showed that among the clones with the H. defensa + PAXS association, the fecundity of clone QC1 was highly significantly reduced by heat (t193 = -6.54, P < 0.0001) while this was not the case for clone QC2 (t193 = -1.51, P = 0.1318) (Fig. 4). For all other clones, fecundity was also greatly reduced by heat stress. The analysis also revealed a significant temperature × symbiont interaction (F3,193 = 3.49, P = 0.0169), indicating differential responses to temperature of symbiont associations in term of fecundity. While a significant reduction in fecundity was observed for all symbiont associations under heat stress, some were more affected than others: aphids harboring H. defensa and H. defensa + PAXS saw their fecundity decreased by 31% and 38%, respectively while those having R. insecticola and S. symbiotica suffered more important reductions, estimated at 47% and 60%.

Fig. 1. Restriction enzyme digestion patterns of PCR-amplified bacterial 16S rDNA from six Acyrthosiphon pisum clones using Sall, which cuts Buchnera aphidicola 16S rDNA in three fragments (53 + 635 + 819 bp; Sandström et al., 2001). As seen on the gel, all clones harbor the obligatory symbiont B. aphidicola (note that only the two largest fragments are detectable). Digestion of PCR products from clones QC1 and QC3 generated two additional fragments (≈500 + ≈1000 bp) that were extracted and sequenced, leading to the identification of PAXS. For all clones, bands of ≈1500 bp correspond to undigested PCR products. Lane M contains a molecular weight marker (1 kb + DNA Ladder).

Fig. 2. Aligned 16S rDNA sequences from different host–symbiont associations, for Escherichia coli nucleotide positions 440–500. A specific 28-bp region present in PAXS and absent in other A. pisum symbionts can be found at E. coli positions 453–480. Dark areas correspond to conserved regions. Abbreviations in parenthesis following the symbiont name refer to the aphid host: AP = A. pisum; CJ = Cinara juniperi; MC = Maculolachnus submacula.
3.3 Resistance of G2 aphids to A. ervi

At dissection to detect the presence of an L1 larva of A. ervi at 96 h post-oviposition, we repeatedly observed for all treatments unviable primary eggs (see Bensadia et al., 2006), specifically among the resistant clones QCV1 and QCV3 harboring both the symbionts H. defensa and PAXS (Fig. 3C). These eggs also showed apparently disorganized early cellularization (see Fig. 3A and B for normal primary egg development in non-resistant clone WIG3). This was not observed in the two other resistant clones, QCR1 and QCVC2, infected with the H. defensa symbiont alone. Under control conditions (T− UV−) at 96 h post-oviposition, aborted primary eggs could be found in about 70% of dissected aphids for the resistant clones QCV1 and QCV3 but not in resistant clones QCR1 and QCVC2.

On the other hand, an L1 larva was found in 85% of non-resistant clones WIG1 and WIG3, indicating that aphids were indeed parasitized and that attacks by female parasitoids resulted in egg laying in the vast majority of cases. A Chi-square test on the proportions of unviable primary eggs among the four resistant clones revealed that their occurrence was strongly correlated to the aphid clone and by extension, its symbiont association ($\chi^2 = 49.4 > \chi^2_{0.001; 3}$). As seen in Fig. 3C, aborted primary eggs could be rapidly confirmed by adding 2.5 μl of 0.4% Trypan blue solution (Sigma, Oakville, ON, Canada) to the dissection buffer, a stain that selectively colors dead tissues (Li et al., 2002; Gantkenbein-Ritter et al., 2008). Subsequent parasitism assays and dissections with resistant clones QCV1 and QCV3 at different time intervals post-oviposition confirmed these results. They also showed that aborted unviable primary eggs of A. ervi could be found up to 10 days post-oviposition under control conditions (T− UV−).

Due to the absence of any variance in parasitism (100% resistance) for clones QCR1, QCVC2 and QCVC1 under control conditions (T− UV−), these three clone–treatment combinations were removed from statistical modeling, as the GLIMMIX estimation procedure failed when they were included (failed convergence of iterative procedure given no variance for these combinations). Similarly, clone QCVC3 remained fully resistant in all treatments and thus was completely excluded from modeling (see Allison, 1999, pp. 39–48; Hosmer and Lemeshow, 2000, pp. 135–142).

The statistical analysis revealed no direct effect of UV-B radiation on A. pisum resistance to A. ervi ($F_{1,131} = 0.00, P = 0.9742$), as well as no interactive effects of any kind (Table 2A). It however exposed a significant temperature × symbiont interaction ($F_{3,131} = 5.44, P = 0.0015$), indicating that the impact of temperature on A. pisum resistance to A. ervi parasitism was related to the facultative bacterial symbionts of the aphid. The effect of different facultative symbionts was examined separately (Table 2B), showing that clonal resistance was affected by temperature in the presence of H. defensa alone ($F_{1,40} = 15.53, P < 0.0001$; clones QCR1 and QCVC2) while it was not affected when H. defensa was associated with PAXS ($F_{1,40} = 3.45, P = 0.0655$; clone QCV1). As seen in Fig. 5, the clones harboring only the H. defensa symbiont became less resistant to A. ervi after exposure to heat, while the two clones also co-infected with PAXS remained highly resistant. Exclusion of clone QCVC3 (H. defensa + PAXS) from modeling does not alter these conclusions, which also applies to clones QCR1, QCVC2 and QCVC1 in the control treatment (T− UV−). In the presence of the other facultative symbionts (S. symbiotica and R. insecticola), the analysis showed that temperature had no significant impact on resistance (Fig. 5).

4. Discussion

4.1 Isolation of PAXS, a new facultative symbiont of A. pisum

PAXS was identified in the two highly resistant clones QCVC1 and QCVC3 (this study) as well as in two other clones available in our laboratory (unpublished data), which were all co-infected with H. defensa. This suggests that this H. defensa + PAXS association may be frequent in the field and can be observed on A. pisum clones collected both on alfalfa and red clover.

Analyses of 16S rDNA sequences showed clear evidence that PAXS is different both from the co-infecting symbiont H. defensa hosted by A. pisum clones in this study, and from the two other common symbionts of A. pisum, S. symbiotica and R. insecticola (Fig. 2). Importantly, close examination of the newly available genome of H. defensa (CP001127) also excluded the possibility that PAXS could correspond to an additional copy of the 16S rDNA gene with a different sequence. The maximum homology of PAXS with all three known H. defensa 16S rDNA operons was 92%. As the minimum accepted similarity threshold for species definition is 97% (Stackebrandt and Goebel, 1994), PAXS can safely be qualified as a previously unreported symbiont of A. pisum. We however found very high similarity between the PAXS 16S rDNA sequence and those of unidentified symbionts of C. juniperi and M. submacula.
These aphid hosts (subfamily Lachninae) are monoeocious, distant relatives of *A. pism* (subfamily Aphidinae), that also exploit distant host-plants: *C. juniperi* is specific to juniper while *M. submacula* is a species feeding on stems and superficial roots of *Rosa* spp. (Blackman and Eastop, 1994, 2006; Normark, 2000). In those aphid species, PAXS-like sequences were however found in absence of *H. defensa*. This suggests that PAXS-like symbionts may be present in a wide diversity of aphids feeding on diverse host-plants, and may associate with aphids independently of *H. defensa*.

4.2. Fecundity of G1 aphids

Heat stress caused a significant reduction in fecundity for almost all *A. pism* clones tested, except QCV3, which is consistent with the findings of previous studies (Ohtaka and Ishikawa, 1991). As expected, the impact of high temperature on fecundity varied greatly depending on the symbiont associations of the aphid host. Globally, aphids harboring *H. defensa* (with or without PAXS) suffered less important reductions in fecundity than those having *R. insecticola* or *S. symbiotica*. This is partly consistent with Russell and Moran (2006) who showed *H. defensa* tendency to confer greater survival to heat-shock, but our results indicate that the protection of aphid fitness in terms of fecundity was not consistent among clones having the same facultative symbionts. Of the two clones with both *H. defensa* and PAXS, QCV1 fecundity was reduced by more than 50% by heat stress, while QCV3 was not significantly affected (Fig. 4). This indicates that fecundity also depends on host genotype irrespective of facultative symbionts, or that different strains of the same symbiont differentially affect fecundity and fitness under heat stress, as suggested by Russell and Moran (2006). Obviously, in the particular case of QCV1 and QCV3, which harbor both *H. defensa* and PAXS, dual facultative symbiosis could greatly increase the range and complexity of possible responses to stress. Our results also indicate that the *S. symbiotica* symbiont commonly associated with heat tolerance in *A. pism* did not contribute to maintain a high fecundity in our infected clone WIG1, which suffered the highest reduction among all heat-exposed clones. Contrary to Chen et al. (2000) and Montllor et al. (2002) whose experiments involved multiple host plants (bur clover, sweet pea and alfalfa), we used broad bean, which is a less limiting host-plant to *A. pism* performance (Ferrari et al., 2008). Russell and Moran (2006) also showed that different strains of *S. symbiotica* confer variable resistance to heat, and suggested that their geographical origin (warmer vs. cooler areas) could be an important factor in explaining this variation. While symbiont associations were not experimentally manipulated here to assess each symbiont effect within a single aphid genotype (clone), these natural infections likely reflect complex aphid geno-type × symbionts genotypes interactions, as evolved under field conditions (Thomas and Blanford, 2003). Further work implicating artificial symbiont infections or curing would be needed to gain a better understanding of the symbionts effects independently of aphid genotypes.

In addition to the genotype of the host and its facultative bacterial symbionts, the obligatory symbiont *B. aphidicola* also plays a major role in aphid performance under heat stress. Dunbar et al. (2007) showed that a mutation in the promoter of the gene *ipbA* that encodes a heat-shock protein in *B. aphidicola* could have a significant impact on the fecundity of *A. pism*. In the absence of stressful conditions, this mutation conferred increased fecundity to the aphid, but it was costly at high temperatures. These authors also showed that this mutation was more common among individuals collected in temperate regions (Wisconsin and New York) than those from warmer regions (Utah and Arizona). It is therefore possible that the clones used in our study, all collected in Québec and Wisconsin, were carrying this mutation, with the possible exception of QCV3, as its fecundity was not reduced under heat stress, and interestingly also had a lower reproductive potential than other clones under cooler temperatures. Whether or not this clone is carrying the *ipbA* promoter mutation, its lower fecundity could alternatively depend on its dual *H. defensa + PAXS* infection, as Oliver et al. (2006) observed that superinfections can be costly to aphid fecundity.

Although fecundity was drastically reduced by heat stress in most cases, very few completely sterile aphids were observed, even after repeated exposure to 35 °C for 4 h daily. These results may seem in conflict with other studies, where an acute stress at around 37 °C for short periods completely sterilized most of the exposed aphids (Ohtaka and Ishikawa, 1991; Russell and Moran, 2006). However, in this study, a possibly more natural heat stress was realized in the form of a fluctuating regime with a daily average temperature of 20 °C, which implied reducing the temperature to 17 °C for 20 h to compensate the 4 h stress at 35 °C. This daily fluctuation in temperature could have helped to reduce the negative consequences of heat stress by providing a long period of recovery to the host and its symbionts. A fluctuating temperature regime may also be more representative of the real conditions observed in the field under temperate latitudes where daily temperature amplitudes between mid-day and late night can be of the same order, therefore implying possible adaptation of the aphids to such fluctuations.

As expected, UV-B radiation adversely affected aphid fecundity, but much less than heat, and independently of clones and types of facultative bacterial symbionts (Fig. 4). These results appear to differ from those of Nguyen et al. (2009), where no direct effects of UV-B were observed on fecundity of the potato aphid *M. euphorbiae* in a similar experiment. Divergence between results of the two studies is probably attributable to a large extent to different intensity of the UV-B stress, which for this experiment was almost double (20.8 kJ m⁻² vs. 11.6 kJ m⁻²).

With a more intense UV-B stress, it is still surprising to find so little effects on *A. pism* fecundity here. Aphids are obviously well protected against UV radiation, despite little melanization of the cuticle, as here for *A. pism*. Several of the pigments identified among Aphididae could play a protective role, and some have been...
shown to have a peak of absorbance in the UV spectrum (Bowie et al., 1966; Kayser, 1985; Walters et al., 1994). These could help preventing UV radiation penetration deep into the aphid and reach the bacteriocytes where are located the majority of bacterial symbionts, and particularly the nutritional symbiont B. aphidicola.

Because abiotic stresses can have both direct and indirect effects (Caldwell et al., 2003, 2007), it is also possible that the observed reduction in fecundity was not entirely linked to a direct physiological stress due to UV-B radiation. Although behavior was not monitored in detail, aphids exposed to UV-B frequently sought to settle, when possible, on the least exposed surface of the leaf discs in Petri dishes, as described by Nguyen et al. (2009) and consistent with the fact that insects can to some extent perceive ambient UV-B (Mazza et al., 2002). Increased movement means less time for feeding, and foraging time is strongly related to fecundity in aphids (Dixon, 1998, pp. 8–26; Nelson, 2007). Thus reduced feeding may be what in fact affected A. pisum fecundity here rather than direct physiological stress caused by UV-B radiation.

4.3. Resistance of G2 aphids to A. ervi

As fecundity reduction on the G1 aphids confirmed that our experimental heat and UV-B stresses were effective, we can therefore assume that the G2 aphids on which resistance to A. ervi was tested were indeed exposed, at least to very substantial heat stress. We found that exposure to UV-B radiation had no impact on the resistance of G2 aphids to A. ervi, contrary to heat stress (Table 2B). In this regard, our results are quite consistent with those of Bensadía et al. (2006), but the present work goes further in revealing the role of a dual facultative symbiosis of A. pisum in preserving the resistance status under heat stress. Among four highly resistant clones hosting H. defensa, only those superinfected clones also harboring PAXS (QCV1 and QCV3) were able to remain resistant under heat stress, in contrast to those singly infected clones with H. defensa (QCR1 and QCV2), that both had their resistance to A. ervi strongly reduced by heat (Fig. 5).

Even without heat stress, the association of PAXS with H. defensa was characterized by particularly strong and very early resistance to A. ervi in clones QCV1 and QCV3, in which even the primary egg of the parasitoid systematically failed to hatch (Fig. 3C). In a non-resistant A. pisum host, the primary egg, which is the first directly observable developmental stage of the parasitoid A. ervi, can be found in the host for a period of time ranging from 0 h to 24 h post-oviposition, after which it hatches into the chorion-less secondary egg, to then pursue its embryonic development into an L1 larva (Grbić and Strand, 1998; Bensadía et al., 2006). We were able to confirm the early death of the unhatched primary eggs in PAXS-infected aphids using Trypan blue staining, thus showing very high resistance to A. ervi in aphids dually infected with PAXS and H. defensa. By contrast, in clones where only H. defensa was present, resistance to A. ervi was still high (inhibition of L1 development in most cases) but it did not express itself as early since primary eggs hatched. We previously showed that A. ervi females do not discriminate A. pisum clones on the basis of immunity to its establishment (Bensadía et al., 2006). The fact that no primary egg was found in aphids with H. defensa but without PAXS shows that development of the early parasitoid embryo went further before being stopped at the more fragile secondary egg stage, which is difficult to observe. The co-symbiont PAXS thus seems to confer additional resistance to A. ervi establishment by inhibiting its development at the earliest possible stage (i.e. the initial cellularization of the chorionated egg), which could reduce the cost of resistance for the aphid host. On a side note, the precocious resistance observed in two A. pisum clones in this study appears to be similar to the findings of Henter and Via (1995) who found primary eggs of A. ervi in resistant A. pisum clones up to 72 h post-oviposition but then apparently disappeared according to the authors. We however observed that aborted primary eggs could still be found in a large proportion at 96 h and even after 10 days post-oviposition, suggesting their long persistence in the highly resistant aphids. Contrary to Henter and Via (1995) who did not report A. ervi primary egg cellular division in resistant hosts, we systematically observed clear cellularization of the aborted eggs (see Fig. 3A vs. C).

Most interestingly, hosting PAXS appears to allow pea aphids to remain highly resistant under heat stress, what H. defensa alone seems unable to do (Table 2B and Fig. 5). This may suggest that PAXS is not affected by heat like H. defensa, or that it is less sensitive, which should be investigated further. PAXS may also contribute to protect H. defensa in the host in a way that may be similar to what S. symbiotica does for B. aphidicola under severe heat stress, by protecting bacteriocytes containing the B. aphidicola symbiont (Montllor et al., 2002).

5. Conclusions

This study extends our knowledge of how abiotic factors, in particular heat and UV-B radiation, affect aphids as symbiotic systems and as hosts to parasitoids. The impact of heat stress on fecundity varied across facultative bacterial symbiont associations, but also among aphid clones harboring similar symbiont types, while UV-B radiation had a negative effect that was consistent among clones, and irrespective of symbiont associations. The main finding of our work was to reveal an ecological role for dual facultative symbiosis under heat stress conditions in pea aphids hosting both H. defensa and the newly discovered facultative symbiont PAXS. Pea aphid clones harboring PAXS in association with H. defensa not only showed more precocious resistance to A. ervi development than aphids harboring the H. defensa symbiont only, but this resistance remained stable under heat stress.

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