Aphid clonal resistance to a parasitoid fails under heat stress

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Abstract

Parasitoid virulence and host resistance are complex interactions depending on metabolic rate and cellular activity, which in aphids additionally implicate heritable secondary symbionts among the Enterobacteriaceae. As performance of the parasitoid, the aphid host and its symbionts may differentially respond to temperature, the success or failure of aphid parasitism is difficult to predict when temperature varies. We tested the hypothesis that resistance of the pea aphid Acyrthosiphon pisum to the parasitoid Aphidius ervi, which is linked to aphid secondary symbionts, may depend on temperature in several resistant and non-resistant aphid clonal lineages of different geographic origin and of known bacterial symbiosis, using experiments in controlled environments. Complete immunity to A. ervi at 20°C in three different aphid clones whose symbiosis is characterized by the possession of Hamiltonella defensa reversed to high susceptibility at 25°C and especially 30°C, suggesting that the aphid’s immune responses to the establishment and early development of the parasitoid is strongly reduced at moderately high temperatures. There was no evidence that a pea aphid control genotype that was susceptible to A. ervi at 20°C could become more resistant as temperature increases, as has been suggested for insect fungal pathogens. By contrast, our results suggest that aphid clonal resistance to A. ervi and related parasitoids is characteristic of cool temperature conditions, similar to various other fitness attributes of aphids. Based on evidence that H. defensa symbionts characterized all three A. ervi resistant pea aphid clones studied, but was absent in control aphids that remained susceptible at all temperatures, we suggest that secondary symbiosis plays a key role in the heat sensitivity of aphid clonal resistance. Our study may also indicate that aphid natural control of variably susceptible host populations by aphid parasitoids is more likely at moderate to high temperatures.

Keywords: Parasitoidism; Aphididae; Braconidae; Aphid clonal resistance; Temperature; Heat stress; Insect immunity; Oviposition; Behaviour; Haemocytes; Symbiosis; Secondary bacteria; Hamiltonella defensa; Serratia symbiotica

1. Introduction

Traits determining the virulence of a parasitoid in a given host, and those determining the susceptibility of the host to the parasitoid are complex behavioural, physiological and cellular attributes (Strand and Pech, 1995; Beckage and Gelman, 2004), which ultimately depend on related genes in the two insects involved. Excepting behaviour, which determines the ability of a wasp to recognize and successfully oviposit in hosts, endoparasitoid virulence is generally mediated by surface attributes of parasitoid eggs and egg-associated biological materials that are injected into the host by the parasitoid wasp. The corresponding traits of hosts are those of physiological immunity, which in insects depend on interactions in the haemolymph between specific classes of haemocytes and molecular defences of the host with early parasitoid stages and especially the egg stage. Insect host immunity and parasitoid virulence are best known in parasitism of caterpillars (Lepidoptera) and Drosophila larvae (Diptera) (Strand and Pech, 1995), but relatively little is known on their environmental mediation. Despite the importance of thermal conditions for insects and the fact that ectothermy potentially constrains parasitoid virulence and host immunity, how temperature is affecting parasitoid vs. host survival is poorly known.

Compatibility of parasitoids and hosts can vary and reverse completely depending on temperature conditions.
(e.g. Fellowes et al., 1999; Blumberg, 1991; Geden, 1997). Similar to fungal infection in insects being prevented or controlled under heat stress, warm or hot conditions could also prevent parasitoid establishment and favour host survival, which has been associated with “behavioural fever” in insects such as grasshoppers (Wilson et al., 2002; Ouedraogo et al., 2003; Thomas and Blanford, 2003). However, insect immunity to parasitoids is not necessarily based on mechanisms similar to those involved in microbial infection.

The objective of this study was to experimentally examine temperature conditions for clonal resistance of aphid hosts (Hemiptera: Aphididae) to their most important parasitoids (Hymenoptera: Braconidae: Aphidiinae). For this purpose, we used the pea aphid *Acrithosiphon pism* (Harris) and the solitary parasitoid *Aphidius ervi* Haliday as a model system.

Parasitism of pea aphid by *A. ervi* is typical of such associations, depending on a brief but initially “mild” parasitic relationship of the parasitoid’s immature stages with the aphid host known as koinobiosis (Askew and Shaw, 1986; Mackauer et al., 1996; Godfray 1994, 2000; Quicke, 1997). *Aphidius* invade hosts as undifferentiated eggs, and develop to the penultimate larva with relatively minor effects on the host, as reflected in continued feeding, growth and metamorphosis, and often a brief period of viviparous reproduction of the aphid (e.g. Cloutier et al., 2000 and references therein). The wasp quickly inserts yolk-deprived or “hydropic” eggs (Strand, 2000) singly in potential aphid hosts with a short ovipositor, where the egg may fail to develop and die at an early stage in aphids that are resistant to the parasitoid (Henter and Via, 1995). A development trait of *Aphidius* that seems to be critical to its overall virulence is the deployment of “giant cells” or “teratocytes” (Quicke, 1997), which genetically are sister cells of the *Aphidius* larva, acting initially as the extra-embryonic membrane of the egg, and later released in the host at larval hatching to continue processing nutrients extracted from the host for larval growth (Falabella et al., 2000). In some pea aphids that are resistant to *A. ervi*, failure of the parasitoid at the larval stage has been linked to incomplete deployment of teratocytes (Li et al., 2002; see also Cloutier and Douglas, 2003), resulting in weak virulence and hence partial host resistance in these interactions.

Moderate-to-high levels of field resistance of pea aphids to *A. ervi* have also been reported in North America and Europe (e.g. Henter and Via, 1995; Hufbauer and Via, 1999; Hufbauer, 2001; Ferrari et al., 2001), where *A. ervi* development failure in resistant aphids occurs before larval hatching, although the resistance mechanisms involved are still unknown. These studies showed that *A. pism* resistance to *A. ervi* in natural aphid populations is variable, possibly related to the host plant of the aphid, and appears to be genetically based, characterizing clonal lineages that result from asexual/viviparous aphid reproduction in the summer in temperate climates. In addition to parasitoid resistance, pea aphid clones typically vary in many qualitative (e.g. colour) and quantitative traits including adaptation to heat stress (Chen et al., 2000; Montllor et al., 2002), although very little is known yet on potential interactions between aphid adaptation to temperature variation, and their adaptation to resistance to parasitoids or pathogens.

Recently, aphid bacterial symbionts within the Enterobacteriaceae (Douglas, 2003a, b; Moran et al., 2005) have been associated with pea aphid resistance to parasitoids, especially *A. ervi* (Oliver et al., 2003, 2005; Ferrari et al., 2004). These “facultative” or “secondary” symbionts are typically associated with large aphid bacteriocytes hosting the primary aphid symbiont *Buchnera* aphidicola (Douglas, 1998; Cloutier and Douglas, 2003) There is now substantial evidence that the secondary symbionts recently described as *Hamiltonella defensa* (previously known as *T = PABS*), and possibly *Serratia symbiotica* (known until recently as R = PASS) are involved in *A. pism* resistance to its parasitoids. Moreover, the *S. symbiotica* symbiont has independently been shown to benefit pea aphid fitness under high-temperature conditions (Chen et al., 2000; Montllor et al., 2002).

In this study, we examined three clonal lines of pea aphid originating from distinct populations in North America, and which exhibit high and heritable resistance to *A. ervi* under standard rearing conditions at 20 °C, to determine if their resistance might be temperature-dependent. In European pea aphid biotypes, Stacey and Fellowes (2002) reported that temperature variation in the range 18–23 °C did not affect pea aphid resistance to *A. ervi* and *A. eadyi*. Here we show that high pea aphid resistance to *A. ervi* expressed at 20 °C fails when the aphid experiences 25 or 30 °C, and this for the three randomly selected North American clones. We show that heat stress affected aphid resistance at the physiological level, and independently of behaviour, which led us to hypothesize that heat-sensitive resistance to parasitoids may be a common characteristic of these aphids from cool temperate climates. Based on PCR determination of the secondary bacterial complement of our aphid lineages, we suggest that heat sensitivity of clonal resistance to parasitoids in pea aphid is most likely linked to the aphid’s symbiosis with Enterobacteriaceae and especially *H. defensa*, which is a variable and widespread trait apparently associated with parasitoid resistance in North American and European populations of *A. pism*.

2. Methods

2.1. Insects

All aphids and parasitoids were reared on broad bean *Vicia faba* under 16L:8D photoperiod at 20 °C in controlled environment cabinets designed for plant growth (Conviron, Winnipeg). To limit interpretation of detected effects to the aphid lineage × temperature interactions, we controlled biological variability of the parasitoid by using *A. ervi* females from a single biotype, which were from
a colony initially established from pea aphid mummies collected on alfalfa in Madison WI. *A. ervi* was introduced during the early 1960s from Europe into the United States for biological control of *A. pisum* (Mackauer, 1969; Hufbauer, 2001). Sometimes described as a generalist, it has most often been associated with *A. pisum* on leguminous crop plants (Mackauer and Finlayson, 1967; Mackauer, 1969), and especially clover (*Trifolium* spp.), and alfalfa (*Medicago sativa*).

The four *A. pisum* clones tested (WIR1, WIR4, QCR1, and QCV2) were established from viviparous individuals randomly collected in spring in pure stands of alfalfa (*M. sativa*) and red clover (*Trifolium pratense*) at the University of Wisconsin (Madison) and at Université Laval (Québec, QC). These clones are characterized by colour (green or pink), geographical origin (Madison WI, USA or Québec, QC), and source host plant (Table 1). Their resistance/susceptibility to parasitism by *A. ervi* under standard conditions (20°C, 16L:8D photoperiod, on *V. faba* host) was first established by preliminary screening. Second- and third-instar nymphs from clones derived from individual, field collected apterous-viviparous adults were each assayed for successful parasitism by *A. ervi* in small cage tests conducted for 15 d, and replicated over several successive generations. Absence or only occasional occurrence of aphid mummification, and wasp emergence under the above standard conditions were considered as evidence of clonal resistance to *A. ervi*. Clones WIR4, QCR1 and QCV2 were thus determined to be highly resistant to *A. ervi*, their resistance remaining high and heritable through at least five generations. In contrast, clone WIR1 was found to be highly susceptible to *A. ervi*, and was included in the temperature tests as a “susceptible control”, to provide evidence that conditions for parasitism by *A. ervi* were highly suitable to successful parasitism under experimental conditions, and at all experimental temperatures.

Specific characterization of the four clones as host to secondary bacteria was based on PCR detection of partial sequences of 16S rDNA (Sandström et al., 2001). Adult aphids of each clone were removed from their host plant for 2 h prior to extraction, and then surface-sterilized by gentle washing in 70% ethanol. Total DNA was extracted from single individuals using the DNeasy Tissue Kit (Qiagen, Mississauga, Ontario) according to the manufacturer’s protocol. Diagnostic PCR was then performed using the primers and standard reaction mix of Sandström et al. (2001), with the reaction conditions being those of Oliver et al. (2003). The whole procedure was repeated twice on different aphid generations, confirming that *H. defensa* is present in WIR4, QCR1 and QCV2, which are *A. ervi* resistant at 20°C, and absent from the temperature-insensitive susceptible clone WIR1 that we used as a control and that was found to be infected by *S. symbiotica* (Table 1).

### 2.2. Experimental procedures

Aphid hosts used in the temperature tests were experimentally reared since birth at a temperature of 20, 25 or 30°C (±1°C) in controlled-environment cabinets, keeping host plant and all other conditions as above. Early second-instar nymphs (N2) of pea aphids from each clone were selected for uniform size, and individually parasitized under direct observation in a Petri dish. A single wasp attack was permitted on each aphid. As biological traits of parasitoid virulence could vary with parasitoid genotype, different, mated female *A. ervi* were used as replicates. Each female wasp was tested on all *A. pisum* clones (C, n = 3 clones), reared at all experimental temperatures (T, n = 3 temperatures, giving a total of 9 C x T combinations). Tests were completed in three different experiments as follows.

**Experiment 1:** This experiment aimed to determine if behavioural rejection of pea aphids by *A. ervi* might also play a role in addition to physiological immunity, in determining pea aphid resistance to parasitism. Aphid colour is known to affect the host acceptance behaviour of *A. ervi* (Battaglia et al., 2000). Oviposition by *Aphidius* generally being completed in 1–2 s, ovipositor contact with an aphid as seen with the eye or stereomicroscope cannot be assumed to automatically result in oviposition. In this experiment, each of 20 *A. ervi* were tested for both host acceptance for oviposition and host susceptibility (lack of physiological resistance), by recording the outcome of two
Fig. 1. Egg stages of the aphid parasitoid *Aphidius ervi*, freshly dissected from resistant and susceptible clones of *Acyrthosiphon pisum*. (A) Chorion-embedded primary egg of *A. ervi* during early cell division, 6h post-oviposition in aphid clone QCV2, which is resistant to the parasitoid. Note several large spherulocytes adhering to egg chorion, which are loaded with green spherules matching the green colour of this aphid clone. (B) Similar contact of pink-coloured pea aphid spherulocytes with 6h old primary egg of *A. ervi* in resistant WIR4 pea aphid, which is pink coloured. (C) Secondary *A. ervi* egg partly hatched from chorion in resistant pea aphid clone QCV2. Note posterior tip of primary egg chorion and aggregate of many green spherulocytes surrounding chorion and secondary egg. Periphery of the secondary egg is delimited by the large, flattened serosal cells. (D) Similar to C, except in resistant pink aphid clone WIR4. (E) Abnormal 30h old, secondary egg of *A. ervi* hatched from chorion in resistant aphid clone WIR4 at 20°C. Note large, rounded serosal cells showing signs of dissociation. (F) Similar aphid clone as (E), except hatched secondary egg is 22h old and apparently normal with well-developed serosal membrane indicating resistance failure at 30°C in WIR4 aphid. Legend: Bla, large blastocysts giving rise to extraembryonic membrane or serosa during early differentiation of *A. ervi* egg; Emb, early embryo of *A. ervi* within serosal membrane; Pec, primary egg chorion; Smc, serosal membrane cells of the secondary egg; Sph, spherulocytes showing coloured globular inclusions. Scale bar = 25μm.
attacks in short sequence, one on each of two different aphids of clones WIR1, WIR4, QCR1 and each temperature (total 18 attacks/wasp, 2 aphids $\times 9T \times 2$ combinations). Of the two aphids, one individual was randomly selected to be dissected within 1 h after the attack, to determine host acceptance by the presence of a parasitoid egg in the aphid haemolymph. The other aphid was saved, returned to its temperature treatment, and dissected 3 or 4 d later when the parasitoid (in susceptible hosts) would have reached the L1-larval stage under experimental conditions.

Experiment 2: To supplement data on the temperature dependence of physiological resistance of clones WIR1, WIR4, QCR1, 16 additional wasps were tested for virulence on one aphid of each $T \times C$ combination (one instead of two aphids per combination). The aphid was dissected 3–4 d post-attack as in Experiment 1. These 16 wasps plus the 20 wasps from Experiment 1 add to $N = 32$ replicate wasps (blocks) that were tested for the effect of temperature on clonal resistance at the physiological level. Only the 20 wasps from Experiment 1 were tested for behavioural host acceptance of the aphids from the four clones.

Experiment 3: This final experiment was conducted separately on clone QCV2, an $A. pisum$ clone different from the ones above, that was also found to be highly resistant to $A. ervi$ in screening tests. Clone QCV2 differs mainly from resistant clones WIR4 and QCR1 in being a green (instead of pink) aphid, and derived from pea aphid field collections on red clover, rather than alfalfa. The resistance of clone QCV2 was tested for temperature dependence using the same procedure as in Experiment 1, with $N = 20$ different $A. ervi$ wasps. Each one was tested on two different host aphids for each experimental temperature, i.e. it was observed to attack six aphids, of which three were used to record host acceptance, and the three other to examine physiological resistance.

2.3. Aphid dissection

All dissections were completed in Pringle’s buffer using fine dissection tools, under a stereomicroscope at magnifications of 40–120×. Over the period of embryonic development, which lasts 3–5 d depending on temperature, an $A. ervi$ egg may be observed under its primary chorionated form which normally lasts 1–1.5 d (Fig. 1), and the secondary or trophamniotic form that follows the extrusion of the early, morula-shaped embryo from the chorion (Grbic and Strand, 1998; Strand, 2000). For simplification, we refer to these two egg stages or forms of the $A. ervi$ egg as the primary egg and secondary egg, respectively. To assert our ability to detect $A. ervi$ secondary eggs that were in trouble in this study because of aphid resistance, preliminary dissections of parasitized aphids were made at 12 h intervals during the period spanning the complete embryonic development (3–5 d) in both susceptible and resistant aphids. In aphids exposed to experimental temperature treatments, $A. ervi$ were mainly observed as fresh primary eggs (~1 h post-attack), or first-instar larvae (L1) hatched from the serosa (dissection 3 or 4 d post-attack). In dissections of aphid hosts 3–4 d post-oviposition, we also searched and counted $A. ervi$ teratocytes in their early growth phase as free cells in the aphid haemolymph, which allowed us to collect data on susceptibility of the aphid to post-larval hatching development of $A. ervi$ at different temperatures. Li et al. (2002) documented a form of apparently nutritional resistance of $A. pisum$ to $A. ervi$, which is characterized by incomplete or suppressed deployment of teratocytes, and which eventually leads to the failure of parasitoid larval development. Despite this form of incomplete resistance not being observed at 20 °C in our pea aphid resistant clones, observations were needed to determine if it might be expressed as temperature was increased to 25 and 30 °C in our experiments. A full complement of teratocytes for $A. ervi$ was considered to be $\geq 20$ cells (Cloutier and Douglas, 2003).

2.4. Data analysis

SAS statistical software PROC GLIMMIX (SAS v. 9.1, http://support.sas.com/rnd/app/da/glmmix.html) was used to test for statistical significance of the clone and temperature (fixed) effects of our design, and of its “block” effect (random) corresponding to replicates with different $A. ervi$ individuals on the variance of the data. The GLIMMIX procedure was used to fit logit models to the data, as suited for binary (presence/absence) data generated by: (i) checking for host acceptance as revealed by the incidence of one $A. ervi$ egg in aphids dissected within 1 h following attack; or (ii) checking for host susceptibility (non-resistance) as revealed by the incidence of an L1 $A. ervi$ larva plus its normal complement of teratocytes in aphids dissected 3–4 d after attack. When logit modelling showed significant clone or temperature effects, the analysis was followed by pairwise comparisons between clones and temperature treatments, using the usual 5% error rate to declare differences as significant.

3. Results

3.1. Host acceptance (Experiments 1 and 3)

Host acceptance of aphids for oviposition was unaffected by temperature ($F_{2,133, df} = 0.30$, $P = 0.7415$) and aphid clone ($F_{2,133, df} = 2.34$, $P = 0.0999$), although the clone effect was close (Table 2A, clones WIR1, WIR4, QCR1). Thus there was no statistical evidence that the 20 female $A. ervi$ reacted to the three different aphid clones as being differently suitable hosts for oviposition, this being true whatever the rearing temperature. This is also confirmed by lack of interaction between the T and C effects ($F_{2,133, df} = 0.71$, $P = 0.5494$). Similarly, separate
analysis of data for clone QCV2 (Table 2A) showed that rearing temperature had no significant effect on aphid acceptance \( (F_{2,38} = 0.4939, \ P = 0.4936) \). Looking at the data shows that oviposition incidence was in the 85–95% range (Fig. 2), indicating that pea aphids from all clones and all rearing temperatures were highly acceptable hosts to the A. ervi wasps, with no evidence that clonal variation in terms of resistance to establishment of the parasitoid was a factor in host acceptance behaviour.

3.2. Temperature effects on host resistance (Experiments 1–3)

Data analysis showed that Clone (Table 2B, \( F_{4,280} = 55.38, \ P < 0.0001 \)) and rearing Temperature (\( F_{4,280} = 23.01, \ P < 0.0001 \)) both had highly significant effects on development of an A. ervi L1 stage. The highly significant Temperature \times\ Clone interaction of the analysis (\( F_{4,280} = 7.03, \ P < 0.0001 \)) further indicates that

![Fig. 2. Host acceptance by the aphid parasitoid Aphidius ervi, as revealed by incidence of oviposition (mean ± SEM) following one attack in Acyrthosiphon pisum susceptible and resistant clones reared at three temperatures. All aphid clones are highly acceptable, irrespective of temperature. Statistical analysis revealed no significant differences between clones or temperatures (see text for details).](image)
temperature effects significantly varied in a clone-specific way. Graphs showing rates of A. ervi establishment and a posteriori statistical comparisons show how each clone performed (Fig. 3). The A. ervi-susceptible clone WIR1 (control) experienced high parasitism under all temperature conditions, the actual rate being slightly higher at 20 than 25°C, with little (non-significant) difference between 30 vs. 20 or 25°C. In clone WIR4, parasitism appeared to increase directly with temperature, being significantly higher at 25 and 30 than at 20°C. In QCR1, data show that parasitism to the L1 stage also increased with temperature but significantly so only under the highest (30°C) condition (Fig. 3). Separate analysis for clone QCV2 (Experiment 3) showed that temperature also had a highly significant effect on A. ervi development to the L1 stage (Table 2B, Fig. 3). L1 hatching incidence rising significantly from nearly zero to above 60% at 30°C. Thus results showed that in all three A. pisum clones that are highly resistant at 20°C, temperature effects were similar, although the milder 25°C heat stress was much more effective in destroying resistance in WIR4 aphids than QCR1 and QCV2 aphids.

### 3.3. Individual variation among A. ervi wasps

There was highly significant variation between A. ervi wasps for acceptance of hosts for egg laying in Experiment 1 ($F_{19,133.6} = 2.57, P = 0.009$) (Table 2A), but insignificant variation among hatching incidence of the L1 larva in progeny of the same females ($F_{35,133.6} = 1.16, P = 0.2499$) (Table 2B). This indicates that controlling for wasp individual variation was useful for assessing the behavioural component of parasitism, i.e. oviposition. That wasp variation no longer appears in the rate of success of parasitism can be explained by lack of, or a lose relation between host acceptance behaviour and actual capacity to establish an egg (virulence) in these A. pisum clones by the wasps in experimental conditions. Data analysis for host acceptance in Experiment 3, which tested aphid clone QCV2 (Table 2A), revealed no inter-individual variation suggesting that in Experiment 1, it might have been induced by the wasps encountering different aphid clones in a short period of time.

### 3.4. Aphid dissection

Hundreds of A. ervi parasitoids in the early stages of egg development and first-instar larval development were observed as a result of both preliminary and experimental aphid dissections. Dissections revealed both primary and secondary eggs (Fig. 1) of A. ervi. The primary egg, which only lasts about 1 d, is easy to detect to its characteristic thin lemon shape (Fig. 1A). The secondary or de-chorionated egg is normally round-shaped and is less observable than the primary egg. When developing normally, it is clearly recognizable to the serosa, which is seen under the microscope as a characteristic outer ring of large, spindle-shaped cells that are flattened on the egg surface at all A. ervi embryonic stages, during and posterior to “hatching” of the primary egg (Fig. 1D). The secondary egg was especially easy to observe during hatching before being completely freed from the chorion. Wasp oviposition (host acceptance) was occasionally confirmed by finding only the empty chorion of the primary egg, which evidently can persist in the aphid after primary egg hatching.

Encapsulation of A. ervi eggs was not observed in resistant pea aphid clones as it is usually described. However, careful dissection frequently revealed small and moderate aggregates of aphid haemocytes on primary and even secondary eggs, including during the relatively long phase of hatching of the primary egg. Based on microscopic examination, the most obvious aphid haemocytes in intimate contact with A. ervi eggs were relatively large ones (~50 μm or more) that carried strongly coloured globules or spherules, which matched the aphid body colour (green, pink), and which we refer to here as spherulocytes (Fig. 1). Obvious clusters of several of these coloured spherulocytes were observed on A. ervi eggs in both resistant and
susceptible pea aphid clones. There was no apparent difference in frequency of such haemocyte aggregation between clones, but the data available are insufficient to provide a full examination of this aspect of pea aphid haemocyte response to *A. ervi* eggs.

4. Discussion

The central question of our study was whether aphid clonal resistance to parasitoids as reported in several studies of the *A. pisum × A. ervi* association in North America and Europe (Henter and Via, 1995; Hufbauer, 2002; Ferrari et al., 2001, 2004) may depend on temperature. As a key factor affecting metabolic performance, temperature could have markedly differential impacts on *Aphidius* virulence vs. resistance of pea aphid hosts, especially considering that aphid bacterial symbionts are implicated in aphid resistance to parasitoids and to heat stress. It is well known that insects can be cured of bacterial infection when exposed to high temperature. The results of our experiments provide strong support to the conclusion that pea aphid clonal resistance to *A. ervi* fails under heat stress, i.e. at temperatures in the 25–30°C range, at least for all the resistant pea aphid biotypes under study here. This was unexpected as Stacey and Fellowes (2002) working with the same aphid attacked by *A. eadyi* found no evidence of aphid resistance failure in the 5°C range of 18–23°C. However, their results are not inconsistent with ours since the susceptibility of our resistant clones failed at 25°C and especially 30°C, suggesting some thermal threshold effect around 25°C. Further work would be necessary to determine temperature thresholds for resistance failure, which may be clone-specific as suggested by significance of the clone effect in data analysis. It is important to note that effective temperatures for resistance failure near 30°C for two of three clones and especially 25°C for the other clone do not represent excessively hot conditions for these aphids of mid-Northern latitudes.

Our data aiming to determine if parasitoid/host behaviour was a factor allow us to clearly exclude clonal-specific rejection/defence behaviour in pea aphid resistance to *A. ervi* in our experiments. Despite significant variation in egg-laying incidence following ovipositor contact (dissection of primary eggs), all clones including highly resistant ones were generally acceptable to many *A. ervi* females. Their variation in immunity to *A. ervi* at 20°C as well as variation in colour, and possibly host plant adaptation, obviously had minor or no effect on the behavioural interaction. It was important to eliminate behaviour even if data were already available on this aspect (Henter and Via, 1995). Oviposition success can vary strongly depending on wasp behaviour during host recognition, approach, and contact with the host (Michaud and Mackauer, 1995; Mackauer et al., 1996; Vökl and Mackauer, 2000). Beyond superficial colour variation and host plant origin, pea aphid clones used here were unknown in terms of relative acceptability to *A. ervi*.

To maximize genotypic homogeneity of *A. ervi*, all wasps were from a single source population, and reared under controlled conditions. For parasitoid rearing, we used a host aphid clone that was distinct from the experimental susceptible control clone (WIRI). We further controlled potential parasitoid-related variation by restricting wasps to only one attack per host for all temperature × host clone combinations, which differs from Henter and Via (1995) who allowed 2–3 ovipositor probes. Our method would control for any possible overcoming of host immunity as a result of multiple oviposition, which cannot be rejected. For solitary endoparasitoids, multiple attack (with or without egg release) might be a potential regulatory mechanism used by wasps to adjust virulence to host susceptibility variation. Here by limiting the number of ovipositor probes to only one, parasitoid sources of variation were strictly controlled. Any remaining variation is attributable to between *A. ervi* wasp individuals, which we included as a random effect in statistical analysis.

Therefore, we conclude that temperature-dependent susceptibility (*A. ervi* hatching and teratocyte deployment) of the resistant clones is best explained by a strongly inhibiting effect of temperature on pea aphid physiological immunity. Reversal of high resistance to high susceptibility occurred in all three resistant clones tested, the effect only being slightly variable between them. Because clones are distinct genotypes (nuclear and symbiotic), each one may have its specific cellular/symbiotic sensitivity to temperature, which when expressed in the physiological functions resulting in immunity to *A. ervi*, produced slightly different effects. There were significant between-parasitoid effects on behaviour (Table 2), but such effects were absent on (post-oviposition) parasitism (lack of effect on L1 incidence, Table 2), which attests to the relative strength of temperature effects on pea aphid physiological immunity.

Behaviour is expected to be more labile/variable than physiological or biochemical traits such as those involved in immunity. We thus suggest that thermo-dependency of parasitoid immunity in the pea aphid may be a widespread characteristic of this species, and possibly other aphids, and that explanations should be sought at the level of the basic mechanisms involved.

Host resistance cannot be understood without also considering parasitoid virulence, which must abrogate, disrupt or defeat host immune responses to be effective (Moreau, 2003; Turnbull et al., 2004). *Aphidius* virulence could possibly be passive and based on egg surface mimicry of host internal tissue, thus avoiding recognition by host immunity factors, as in other parasitoids (Vinson, 1990; Strand and Pech, 1995). Or it could be based on active fighting or repelling aphid immune responses, with factors originating from wasp venom and/or the expression of injected polyDNA viruses (Quicke, 1997; Beckage and Gelman, 2004). Additional virulence factors could also be released by the *A. ervi* egg and early larval stages, as products of the egg serosal cells differentiating at a very early stage in Aphidiinae (Tremblay and Caltagirone, ...
and later by serosa-derived teratocytes, which disperse in the host at larval hatching. However, the issue of parasitoid virulence is probably not important here because parasitoid factors were under stringent control by experimental design, and factoring parasitoid variation in data analysis (Table 2). Therefore, temperature dependency of resistance in pea aphid most likely relates to the (clone-specific) thermo-sensitivity of the aphid’s immunocompetence, which should depend on interaction of nuclear-based aphid physiology with bacterial symbiosis.

Based on our observations, we suggest that high temperatures most likely reduced aphid immunity by preventing critical steps of disruption of organization and hatching of the parasitoid secondary egg. For maximum temperatures most likely reduced aphid immunity by nuclear-based aphid physiology with bacterial symbiosis, coma, which should depend on interaction of (clone-specific) thermo-sensitivity of the aphid’s immunocompetence. Aphid haemocyte aggregates on A. ervi eggs were frequently observed in all aphid clones investigated. They typically involved the recruitment of several individual large aphid cells on the A. ervi egg (Fig. 1), but only partial coverage of the egg was observed. However, very intimate cell surface contact was quite characteristic, sometimes with obvious cell spreading on the parasitoid egg chorion. To our knowledge these interactions with target parasitoid eggs and that we described as haemocyte aggregation, have not been reported before. We hypothesize that they play a key role in pea aphid cellular immunity, and that in combination with symbiosis, they are involved in its physiological resistance to A. ervi and possibly other aphid parasitoids, although their precise role remains to be determined.

Physiological immunity to parasitoids is not described for aphids, in contrast to some major groups of typical hosts to parasitoids. In Lepidoptera, immunity is based on cooperation of specialized haemocytes as markers and killers of biotic invaders, especially granulocytes, plasmaocytes and spherulocytes (Pech and Strand, 1996; Stettler et al., 1998; Lavine and Strand, 2002), which collaborate to fight parasitoids and microbes. Immunity to parasitoids is also well documented in Drosophila (Carton and Nappi, 1997; Russo et al., 2001; Lavine and Strand, 2002; Carton et al., 2005) in which lamellocyte populations encapsulate early parasitoid stages. For many insects that resist parasitoids, encapsulation is the best-documented haemocyte-dependent process associated with immunity to endoparasitoids (Strand and Pech, 1995; Turnbull et al., 2004). However, the numerous studies and reports of pea aphid resistance to Aphidius (Henter and Via, 1995; Hufbauer and Via, 1999; Ferrari et al., 2001, 2004; Hufbauer, 2001) encapsulation per se has never been reported in parasitized aphids, which is confirmed again in the present study.

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However, as reported in the results section, we frequently observed aphid haemocytes on eggs of A. ervi, and especially, aggregates of the large pigment-loaded cells that we referred to above as spherulocytes (Fig. 1). They were observed especially on the chorion of primary eggs of A. ervi, and also around hatching-primary and early secondary eggs. Aphid haemocytes have rarely been investigated (but see Boiteau and Perron’s (1976) report on Macrosiphum euphorbiae). We did not attempt to categorize A. pisum haemocytes, which would require additional investigation. However, at least one large morphotype of pea aphid haemocyte was consistently observed in very intimate contact with A. ervi eggs. Their categorization as spherulocytes is tentative, aiming to refer particularly to their conspicuous cell contents, consisting mainly of abundant large vesicles or “spherules”, which characteristically were either pink or green in fresh dissecta (Fig. 1), and hence closely matched the aphid host’s body colour itself.

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Mechanistically relating aphid immunity to parasitism with temperature obviously will require additional work, although it is already clear that aphid bacterial symbionts play an important role in these phenomena. Secondary pea aphid symbionts are most characteristic of syncytial tissue enveloping the aphid mycetomes, but have also been found in other aphid cellular compartments, noticeably in pea aphid haemocytes (Moran et al., 2005). Oliver et al. (2003) first produced experimental evidence that symbiont H. defensa (T = PABS) and to a lesser extent symbiont S. symbiotica (R = PASS) confer resistance to A. ervi. Ferrari et al. (2004) were able to correlate secondary bacteria with parasitoid resistance in many pea aphid clones collected in south England. Using T-RFLP to screen clones for several major pea aphid secondary symbionts, they confirmed the association of PABS(T) ( = H. defensa) but not PASS (R) ( = S. symbiotica) with parasitoid resistance, actually reporting a stronger correlation with A. eadyi resistance than A. ervi. Recently, Oliver et al. (2005) found further evidence of the major role of H. defensa in conferring partial resistance to A. ervi in North American pea aphid, by examining parasitism of different aphid genotypes experimentally infected with different isolates of H. defensa.

Independently, temperature work on pea aphid symbiosis (Chen et al., 2000; Montllor et al., 2002) suggests that infection with S. symbiotica (R = PASS) can protect the Buchnera-loaded bacteriocytes of the aphid host during heat stress, and that S. symbiotica incidence peaks during hot summer conditions. Previous work had shown that temperature has complex effects on the nutritionally essential Buchnera symbiosis, with severe heat stress being highly damaging (Ohtaka and Ishikawa, 1991; Humphreys and Douglas, 1997).

Based on all available evidence, we hypothesize that temperature effects on A. ervi-resistance as observed in our pea aphid clones are indirectly mediated by secondary bacteria. Significant inter-clonal variation, as evidenced in data analysis, could result from slightly different protection levels afforded by different levels of secondary symbioses,
interacting with different pea aphid nuclear-\textit{Buchnera}, and secondary symbiotic genotype associations. If the primary \textit{Buchnera} symbiosis of the pea aphid is particularly heat-sensitive (Ohtaka and Ishikawa, 1991; Wilcox et al., 2003) but may be rescued by \textit{S. symbiotica} (Chen et al., 2000; Montllor et al., 2002), and if \textit{H. defensa} can protect the aphid from parasitism (Oliver et al., 2003, 2005), therefore the possession of both secondary symbionts might help aphid-\textit{Buchnera} associations to survive parasitism in conditions of heat stress. Work is currently underway to better characterize the role of secondary bacteria in our resistant and non-resistant pea aphid clones, in order to empirically address the secondary symbiont hypothesis in relation to failure of \textit{A. pisum} resistance to \textit{A. ervi} under heat stress.

Substantial additional experimental work may be necessary to clarify these relationships since the overall effect of multiple bacterial symbioses (possibly implicating up to five symbiotic bacteria in pea aphid) is impossible to predict by extrapolation from simple to multiple secondary symbioses. Independently both secondary bacteria may appear as useful additional associates that could protect the aphid and its \textit{Buchnera} from distinct stresses (heat and incipient parasitism), and thus contribute further to the independent fitness of each bi-symbiotic association. However, without evidence that their direct interaction has additive or perhaps synergistic beneficial effects on aphid fitness, their joint effect on the primary \textit{Buchnera–A. pisum} symbiosis cannot be predicted. Because all aphid symbionts ultimately depend on the aphid host as the phloem sap collector and basic resource supplier, competition between them for host cellular compartments and basic metabolic resources is most likely, and could only be relieved by mutualism.

As ectotherms adapted to live in intimate relationship with vascular plants, the behaviour of aphids in relation to solar radiation and air temperature variation should be useful for predicting how temperature and climate mediate natural enemy impact, which depends on the full expression of aphid immunity as necessary for \textit{A. pisum} to survive attack by \textit{Aphidius} wasps on plants. If parasitized aphids can be manipulated by parasitoid larvae to maximize parasitoid survival (Lagos et al., 2001; Brodeur and Boivin, 2004; see also Chow and Mackauer, 1999), then aphids detecting parasitoid activity and especially those aphids that have just been attacked by a parasitoid wasp should maximize their immune response by seeking cooler locations on their host plants.

Aphid diversity is greatest in temperate regions of the world (Dixon, 1998, p. 246) and as many other aphid fitness traits, aphid resistance to parasitoids may optimally function only under relatively cool conditions, thus being inherently subject to failure under heat stress. Interestingly, the frequent pattern of aphid population crash in mid-summer has been attributed among major ecological factors to increased parasitoid pressure (Karley et al., 2004 and references therein). As suggested above, temperatures in the 25–30°C are not particularly hot, but in our experiments they were applied on a full-time 24 h basis, rather than the natural day–night, sine wave pattern of fluctuation. Our results might suggest that in cool climates such as in the St-Lawrence or Great Lakes regions of North America, aphid parasitoid mortality should have disproportionate impact on aphids precisely under hot summer conditions, rather than earlier/later in the season. A more specific prediction could emerge if we knew how temperature interacts with exposure time to affect aphid resistance and bacterial symbiosis, and what is the overall effect on the extent of clonal variation of thermal limits for parasitoid resistance within aphid populations, and between different aphid populations.

5. Conclusions

1. We experimentally examined the effect of temperature (20, 25 and 30°C) on the expression of physiological resistance to the parasitoid \textit{A. ervi} in highly resistant pea aphid genotypes represented by clonal lineages originating from different host plants and localities of North America, and with known associations with aphid secondary symbionts \textit{H. defensa} and \textit{S. symbiotica}.

2. Young-mated \textit{A. ervi} females quickly laid eggs in > 80% of aphids of all \textit{A. pisum} clones, with no relation to their resistance to the parasitoid. Behaviour was not a determining factor in observed clonal variation of resistance to \textit{A. ervi}, despite statistical evidence of significant inter-individual wasp variation in acceptance of variably susceptible aphid clones for oviposition.

3. Temperature had a strong effect on the expression of pea aphid clonal resistance to \textit{A. ervi}, as all three randomly selected clones with efficient and heritable resistance at 20°C became highly susceptible at 25 or 30°C. There was no evidence that susceptibility to parasitism at 20°C can switch to partial or complete resistance at higher temperature as reported for aphid susceptibility to fungal pathogens.

4. As many aphid fitness traits, \textit{A. pisum} resistance to its main aphidine endo-parasitoid appears to be dependent on cool conditions. Available evidence suggests that temperature sensitivity of this trait in pea aphid is most likely mediated by complex interactions involving nuclear-based cellular immunity and bacterial symbiosis-related factors.

5. If heat sensitivity is a general feature of immunity to parasitoids in natural aphid populations, the impact of parasitism should be higher under hot ambient conditions, as in the mid-summer.

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