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SHORT COMMUNICATION

Grain aphid clones vary in frost resistance, but this trait is not influenced by facultative endosymbionts

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Abstract. 1. Facultative endosymbiotic bacteria of insects are known to affect life-history traits of their hosts, and can provide important fitness benefits under certain environmental conditions. While several distinct endosymbiont-induced effects have been reported, there is no data on whether heritable facultative endosymbionts in any species affect their hosts' performance at low temperatures, something that could have a major effect on insect physiology and survival, and thus population structure and distribution.

2. The original facultative endosymbionts were experimentally removed from five clonal genotypes of the grain aphid, *Sitobion avenae* Fab., which were then exposed to frost.

3. Aphid genotypes differed considerably in survival following the exposure and in fecundity of the survivors. However, the presence of the facultative symbionts had no overall effect on the studied traits.

4. The results suggest that the facultative symbionts have limited effects on the cold hardiness of their grain aphid hosts.

Key words. *Acyrthosiphon pisum* pea aphid, cold hardiness, *Hamiltonella defensa*, *Regiella insecticola*, secondary endosymbionts, *Sitobion avenae* grain aphid.

Introduction

Facultative endosymbionts of insects have been shown to have a wide range of effects on the life-history traits of their hosts, and there is a growing understanding of their role in insect ecology and evolution (Moran *et al*., 2008; Oliver *et al*., 2010). In aphids, a model system for the study of this type of symbiosis, the benefits of infection with facultative endosymbionts include resistance to natural enemies, protection against abiotic stressors such as heat shock, and improved performance on particular host plants (reviewed by Oliver *et al*., 2010). At the same time, the costs of infection in clones naturally carrying symbionts appear to be low or absent (e.g. Leonardo, 2004; McLean *et al*., 2011), making it unclear why endosymbionts are not more widespread across aphid species and clones. However, with the exception of work on heat shock resistance (Montllor *et al*., 2002; Russell & Moran, 2006), most studies on aphid endosymbionts have been conducted under benign laboratory conditions and the costs and benefits of carrying endosymbionts under fluctuating and often stressful natural conditions are not known.

One of the most important factors determining the population structure and distribution of insects in temperate zones is periodic exposure to sub-zero temperatures (Bale, 2002). Frost can lead to tissue damage resulting from ice crystal formation within cells with major effects on insect survival and fecundity (Parish & Bale, 1993). Insects have evolved numerous mechanisms to enable them to withstand low temperatures (Bale, 2002; Doucet *et al*., 2009), but their cold hardiness can also be influenced by the microorganisms with which they are associated. Gut and surface bacteria have been shown to act as ice nucleating agents increasing the freezing point of tissue in overwintering insects (Lee *et al*., 1992, 1993) and therefore leading to higher mortality in frost-susceptible species. Facultative endosymbionts may also be capable of producing, or affecting the production of, compounds such as antifreeze proteins, polyols and sugars, ice nucleating proteins or heat shock proteins, which help to prevent or control ice crystal growth and reduce frost-related damage, or aid their hosts' recovery following freezing (Bale, 2002; Doucet *et al*., 2009;

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Burke *et al*., 2010; Neelakanta *et al*., 2010). Insect fitness can be reduced if their obligate nutritional endosymbionts are damaged by extremes of hot or cold (Ohtaka & Ishikawa, 1991; Parish & Bale, 1991), and genetic changes in the obligate symbionts can increase their susceptibility to heat shock, so affecting the thermal tolerance of their insect hosts (Dunbar *et al*., 2007). However, facultative endosymbionts can provide some protection following heat shock (Montllor *et al*., 2002; Russell & Moran, 2006). Symbionts may also affect their hosts' survival during the cold season indirectly – by altering their behaviour, or influencing the induction of the sexual generation which produces frost-resistant eggs in aphids (Leonardo & Mondor, 2006). Finally, the beneficial effects of infection with facultative endosymbionts in aphids, such as provision of resistance to natural enemies, are known to vary with temperature (Bensadia *et al*., 2006; Guay *et al*., 2009). Regardless of the mechanisms involved, the prevalence of the symbiont *Regiella insecticola* in pea aphids collected in colder, drier parts of Japan suggests that the infection might be beneficial under cooler conditions (Tsuchida *et al*., 2002). However, despite the many ways in which responses to temperature can be influenced by symbiont infection status, we are not aware of any study that has specifically asked whether maternally transmitted facultative endosymbionts affect their insect host's sensitivity to low temperatures.

Frost sensitivity has received considerable attention in the grain aphid *Sitobion avenae* Fab. 1775, a serious pest of cereals in Europe (e.g. Knight & Bale, 1986; Parish & Bale, 1991; Powell & Bale, 2004). In regions with severe winters, populations of *S. avenae* are largely holocyclic (i.e. alternating parthenogenesis with sexual reproduction) and overwinter as frost-resistant eggs (Papura *et al*., 2003). However, in regions with milder winters anholocyclic (obligatorily asexual) clones overwinter as feeding nymphs and adults, although they can frequently be exposed to sub-zero temperatures and suffer high mortality (Dewar & Carter, 1984; Knight & Bale, 1986; Dedryver *et al*., 2001). In the south of England, grain aphid populations are mostly composed of anholocyclic clones (Newton & Dixon, 1988; Llewellyn *et al*., 2003), which vary in their susceptibility to cold (Griffiths & Wratten, 1979). Grain aphids in this region frequently harbour two species of facultative endosymbionts, *Hamiltonella defensa* and *Regiella insecticola* (P. Łukasik, unpublished), but the symbionts' effects on ecologically important traits in this species are unknown. The present study tested whether the cold hardiness of *S. avenae* can be affected by their facultative endosymbionts. Five genetically distinct clones of *S. avenae* that were naturally infected with *H. defensa* or *R. insecticola* were selected and lineages cured of the infection were created using antibiotics. The mortality due to freezing and the fecundity of aphids that survived the exposure to frost were compared in each pair of infected and cured lineages.

Materials and methods

The clonal genotypes of *S. avenae* used in this study were collected in June 2008 from wheat on an organic farm near Faringdon, Oxfordshire, southern England, where the grass temperatures drop below −10 °C during most winters (Radcliffe Meteorological Station, 2011). The aphids were cultured in 90-mm non-vented Petri dishes at 20 ± 1°C and LD 16:8 h light regime on regularly exchanged, approximately 10-day old wheat plants with their stems placed in 2% agar. These conditions ensured indefinite asexual reproduction. Five clones of *S. avenae* (distinct at seven microsatellite loci; see Tables S1 and S2 for details) were selected for the study, including four genotypes (code numbers Co08, Co23, Co26, and Co37) originally infected with *H. defensa* and one (Co21) originally infected with *R. insecticola*. Polymerase chain reactions with diagnostic primers revealed none of the additional facultative microbial symbionts reported from other aphid species, including *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma*, and *Rickettsiella* (McLean *et al*., 2011; Tsuchida *et al*., 2010), and confirmed the presence of the essential primary endosymbiont *Buchnera aphidicola*. Not less than 12 generations before the experiment, facultative symbionts from the experimental genotypes were removed by the oral administration of antibiotics (2% ampicillin for Co21 and Co26, or a mixture of 0.5% ampicillin, 0.7% gentamicin and 0.3% cefotaxine for Co08, Co23, and Co37) through young wheat plants, at doses that did not eliminate *Buchnera* (McLean *et al*., 2011). Successful elimination of facultative symbionts was confirmed with PCR in not less than four separate generations for each genotype. The resulting five pairs of lineages, sharing genotype and primary symbiont but differing in the presence of secondary symbionts, were used for assessing the effects of symbionts on frost resistance.

The post-freezing mortality of 200 nymphs from each of the 10 experimental lineages was assessed. First-instar experimental aphids aged 36 ± 12 h were transferred in groups of 20 to dishes containing young wheat plants with their stems inserted into parafilm-sealed Eppendorf tubes containing water. After the aphids settled on the plants, the dishes were randomly arranged inside an incubator (Model 1200, LMS Ltd, Sevenoaks, Kent, UK), which was cooled from 20 to 0 °C at a rate of 0.25 $^\circ\mathrm{C}\ \mathrm{min}^{-1}$ and then maintained at that temperature for 3 h before being cooled at the same rate to −10 °C. Six hours later (after 5 h 20 min at −10 °C), the dishes were rewarmed to 20 °C, again at a rate of 0.25 $^\circ\mathrm{C}\ \mathrm{min}^{-1}$. Fresh plants were then added to each dish and after 48 h at 20 °C the proportion of aphids surviving was assessed. All insects found feeding or walking around the dish were classified as surviving (Powell & Bale, 2004).

To assess the effect of freezing on fecundity, groups of aphids aged 36 ± 3 h were subjected to a similar treatment to the above, except that the exposure to −10 °C lasted for only 2 h 20 min. After the end of the cold treatment the aphids were kept for 48 h and then 12 survivors from each experimental lineage were randomly selected from the full set of dishes and kept isolated in Petri dishes on wheat plants which were exchanged every 3 days, with the offspring counted after each exchange. Simultaneously, the fecundity of the same number of control aphids, kept at 20 °C and not exposed to frost, was measured. The number of offspring produced in the first 15 days of life by females surviving until the end of the study was used as a measure of fecundity. Only data for

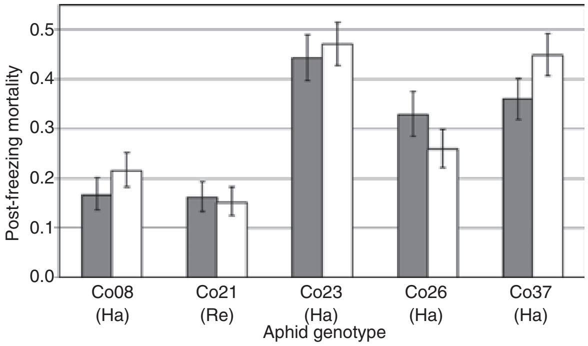

Fig. 1. Mortality (mean ± standard error) of juveniles from five genotypes of *Sitobion avenae,* originally infected with facultative symbionts *Hamiltonella defensa* (Ha) or *Regiella insecticola* (Re) (grey bars) or cured from the infection (white bars), recorded 48 h after the end of a 6-h exposure to -10° C.

wingless individuals was used. There was no clear pattern of the occurrence of winged individuals across treatments or aphid lines.

Data were analysed using the statistical package R v. 2.9.2 (R Development Core Team, 2011) using generalised linear modelling techniques with aphid genotype, symbiont infection status, and treatment (fecundity assay only) as explanatory variables. In order to correct for overdispersion in the data, quasi-binomial (survival) or quasi-Poisson (fecundity) error variances were assumed.

Results and discussion

The mortality following freezing differed significantly between the five experimental genotypes ($F_{4,90} = 22.23$, $P < 0.001$). However, the presence of the facultative symbionts did not affect aphid survival ($F_{1,86} = 0.59$, $P = 0.44$), and there was no aphid genotype by symbiont presence interaction ($F_{4,85} = 1.09$, $P = 0.37$) (Fig. 1). Similarly, the aphid genotypes differed significantly in their overall fecundity ($F_{4,164} = 9.21$, $P < 0.001$) and in the effect freezing had on their fecundity (genotype by treatment interaction: $F_{4,158} = 5.12$, $P < 0.001$), but aphid fecundity was not significantly affected by the presence of endosymbionts ($F_{1,159} = 2.10$, $P = 0.15$) and there was no significant interaction between endosymbiont presence and treatment ($F_{4,154} = 1.93$, $P = 0.11$) (Fig. 2).

Significant differences were found amongst the experimental clones of *S. avenae* in their susceptibility to frost, as revealed both by differences in post-freezing mortality and in the survivors' fecundity. Such clonal variation has previously been reported in *S. avenae* and in other aphid species (Griffiths & Wratten, 1979; Vorburger, 2004). However, no evidence was found that any of the facultative endosymbiont strains used in this study had a significant effect – either positive or negative – on the fitness of their aphid hosts following this brief exposure to sub-zero temperatures. These results indicate that the facultative symbionts infecting grain aphids, *H. defensa* and *R. insecticola*, are not a major force shaping the structure of anholocyclic aphid populations in response to freezing.

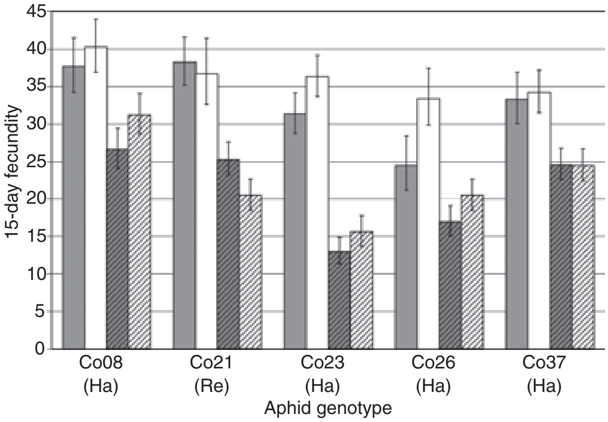

Fig. 2. Numbers of offspring (mean ± standard error) produced within 15 days by wingless females from five genotypes of *Sitobion avenae*, originally infected with facultative endosymbionts *Hamiltonella defensa* (Ha) or *Regiella insecticola* (Re) (grey bars) or cured from the infection (white bars), and either kept at a constant temperature of 20 °C throughout the study (darker striped bars), or exposed as juveniles for 3 h to −10 °C (lighter striped bars).

The absence of apparent costs of carrying symbionts in naturally infected clones under permissible conditions has been noted previously in the pea aphid (Leonardo, 2004; McLean *et al*., 2011) but not in other aphid species. However, facultative symbionts rely on nutrients provided by the hosts for their upkeep, incurring some costs to their carriers, which need to be compensated for in order for the infection to persist in a population. The five experimental symbiont strains do not appear to protect grain aphids against their natural enemies (P. Łukasik & H. Guo, unpublished), and thus the prevalence and diversity of facultative endosymbionts in grain aphid populations need other explanations. Further work on the effects of facultative endosymbionts on thermal sensitivity of their hosts (Montllor *et al*., 2002; Russell & Moran, 2006) and on temperature dependency of other fitness effects of infection (Bensadia *et al*., 2006; Guay *et al*., 2009) will be essential for understanding how symbiosis in insect species and communities varies across climatic zones.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2311.2011.01321.x

Tables S1–S2. Microsatellite markers used to distinguish the grain aphid clones used in the study and the microsatellite profiles of these clones.

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