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Short-term heat stress results in diminution of bacterial symbionts but has little effect on life history in adult female citrus mealybugs

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Abstract

Mealybugs are sap-feeding insect pests that pose a serious threat to horticulture. The citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), like most other mealybug species, harbours two obligate maternally transmitted bacterial endosymbionts, which are essential for nutrient acquisition and host survival. These are '*Candidatus* Tremblaya princeps', a member of the β-Proteobacteria, and '*Candidatus* Moranella endobia', a member of the γ-Proteobacteria. The density of symbionts in the hosts is now understood to be dynamic, being influenced by the age and gender of the host and by environmental conditions during development. Here, we examine the impact of short-term heat stress treatment on the obligate symbionts and life-history parameters of *P. citri*, using qPCR to measure changes in symbiont density. Heat stress killed juveniles and adult males, and significantly reduced levels of '*Ca.* Moranella endobia' and '*Ca.* Tremblaya princeps' in adult females. However, adult females were resilient to this and it did not affect their fecundity or brood survival, although the sex ratio of their brood was slightly, but significantly, more female biased. Our results suggest that '*Ca.* Tremblaya princeps' and '*Ca.* Moranella endobia' are not as essential to the survival of adult mealybugs as they are to the survival of immature mealybugs and that sub-lethal heat treatment alone is unlikely to be effective as a disinfestation tactic.

Introduction

Bacterial endosymbiosis is now appreciated to be a diverse, integral, and influential aspect of insect ecology and evolution (Saffo, 1992), which has potential applications in sustainable pest management, known as 'microbial resource management' (Douglas, 2007; Verstraete et al., 2007; Read et al., 2011; Crotti et al., 2012). Many insects harbour obligate bacterial symbionts which are essential for their survival, but the prevalence and density of symbionts is often dynamic, being influenced by the age and gender of the host and environmental conditions (Chiel et al., 2007; Kono et al., 2008; Moran et al., 2008; Burke et al., 2010).

Mealybugs (Hemiptera: Sternorrhyncha: Pseudococcidae) comprise around 2 000 species worldwide (Thao et al., 2002). These sap-feeding pests pose a persistent threat to horticulture due to their mechanical damage, the transmission of a range of plant pathogens, and the excretion of honeydew which encourages the growth of black sooty moulds (Jelkmann et al., 1997; Sether et al., 1998; Charles et al., 2006). The citrus mealybug, *Planococcus citri* (Risso), is one of the most economically destructive species of mealybug, being a polyphagous and cosmopolitan pest that can feed upon plants from dozens of families (Ben-Dov, 2013) including citrus, cocoa (Ackonor, 2002), coffee (Staver et al., 2001), grapevine (Cid et al., 2006), and other horticultural and ornamental crops in greenhouses and conservatories (Brødsgaard & Albajes, 2000; Laflin & Parrella, 2004). *Planococcus citri* is an international pest, native to Asia, but occurring across the tropics, Europe, Oceania, USA, and Mexico, at outside temperatures ranging from 20 to 32 °C, or in greenhouses (CABI/EPPO, 1999).

Planococcus citri transmits plant pathogens such as grapevine leafroll-associated virus 3 (GLRaV-3) (Cid & Fereres, 2010), badnavirus (Phillips et al., 1999), vitivirus

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(Adams et al., 2004), piper yellow mottle virus (Lockhart et al., 1997), and ampelovirus (Martelli et al., 2002). Chemical application is the most common control strategy of mealybugs (Franco et al., 2009); however, they are difficult to eliminate due to their cryptic behaviour and waxy secretions which shield them from pesticides. Biological control strategies have been explored, including parasitoids, predators, nematodes, and fungi, with mixed results (Odindo, 1992; Stuart et al., 1997; Davies et al., 2004; Ceballo & Walter, 2005; Afifi et al., 2010; Demirci et al., 2011; van Niekerk & Malan, 2012). More effective and reliable strategies are needed.

Planococcus citri, like most mealybug species, harbours two obligate maternally transmitted bacterial endosymbionts within the bacteriome. These are '*Candidatus* Tremblaya princeps' and '*Candidatus* Moranella endobia', the latter residing within the former, a feature believed to be unique to the Pseudococcidae (von Dohlen et al., 2001; Thao et al., 2002; Keeling, 2011; McCutcheon & von Dohlen, 2011). The mutualistic relationship between *P. citri* and these symbionts likely evolved because of the restricted diet of the host, a common characteristic in insect-endobacteria relationships (Douglas, 2006). Mealybugs feed solely upon plant sap, which is deficient in essential amino acids that the insect cannot assimilate. Endosymbionts can compensate for these shortfalls with their wider metabolic capacity and thus provide nutrients for the hosts, allowing them to exploit otherwise impenetrable niches (Douglas, 2009). '*Candidatus* Tremblaya princeps' and '*Ca.* Moranella endobia' are capable of synthesising the full range of required essential amino acids through a fusion of genetic pathways (Keeling, 2011; McCutcheon & von Dohlen, 2011; Husnik et al., 2013). This biochemical complementation demonstrates the evolutionary specificity of these partners and why no successful in vitro culturing nor aposymbiotic mealybugs have been reported.

The ecological function of '*Ca.* Tremblaya princeps' and '*Ca.* Moranella endobia' may lead to variations in the level of infection based upon host requirements. The abundance of '*Ca.* Tremblaya princeps' and '*Ca.* Moranella endobia' alters, depending upon the age and gender of the host (Kono et al., 2008). A qPCR study into these dynamics with the mealybugs *Planococcus kraunhiae* (Kuwana) and *Pseudococcus comstocki* (Kuwana) found that, although females maintain their endosymbionts after maturity, they are at reduced levels and males lose their endosymbionts entirely after pupation, most likely because adult males do not feed (Miller, 1999) and therefore do not require nutritional symbionts. Despite their physical and biochemical connections, this loss of symbionts is decoupled, with '*Ca.* Moranella endobia' disappearing more quickly than '*Ca.* Tremblaya princeps' in males (Kono et al., 2008).

Rearing temperature influences the life-history parameters of mealybugs. *Planococcus citri* instars died below 12 °C and above 37 °C, and the longevity of adult females is greatest at 18 °C, whereas fecundity is highest at 23 °C (Goldasteh et al., 2009). A constant temperature of 30 °C as opposed to 25 °C led to female-biased sex ratios in *P. citri* (Ross et al., 2011), whereas another study found sex ratios to be female-biased at 15–30 °C, but male-biased at 32 °C (Goldasteh et al., 2009). Older mating ages and starvation also triggered this male bias (Varndell & Godfray, 1996; Ross et al., 2011). Other species of mealybug show similar life-history patterns, with fecundity, longevity, and adult weight peaking at species-specific optimum temperatures in *Maconellicoccus hirsutus* (Green) (Patil et al., 2011), *Pseudococcus citriculus* (Green) and *P. kraunhiae* (Arai, 1996), *Paracoccus marginatus* Williams & Granara de Willink (Amarasekare et al., 2008), and *Pseudococcus longispinus* (Targioni Tozzeti) (Santa-Cecília et al., 2011). Long-term exposure of juvenile and adult *P. citri* to 39 °C led to dismantling of the mycetocytes, ultimately leading to the death of the hosts (Köhler & Schwartz, 1962). This demonstrates temperature as a limiting factor in mealybug growth and reproduction, and as an influential factor in sex determination.

Short-term heat stress treatment has been found to lead to dramatic reductions in obligate symbiont density in the pea aphid *Acyrthosiphon pisum* (Harris), with an observed 80% loss of the bacterium *Buchnera aphidicola* Munson et al., which did not recover 96 h following treatment, unless the host was co-infected with the facultative symbiont *Serratia symbiotica* Moran et al. (Burke et al., 2010). It has yet to be studied whether short-term temperature stress could lead to the reduction in symbionts in mealybugs, or distort the life history and/or sex ratio in a way that is sub-optimal for their population regeneration, which could potentially be applied as a pest control tactic. Here, we examine the impact of short-term heat stress on the symbionts and life-history parameters of *P. citri*, using qPCR to measure changes in symbiont density.

Materials and methods

Sourcing and rearing of mealybugs

Individual *P. citri* were collected from the horticultural research centre Proefcentrum voor Sierteelt, Ghent, Belgium. These were sourced from a variety of host ornamental plants which had been brought in from commercial greenhouses from across Belgium and pooled into a single laboratory population. Mealybugs were cultured in darkness at 25 °C and 50% r.h. on white organic potato sprouts. Offspring from this established 16-month-old laboratory population were used in the experiment.

Mealybug eggs laid by multiple females were collected and reared for 29 days until females had reached maturity, with pupating males being separated from females to ensure female virginity.

Heat stress treatment

At the end of the rearing period, half of the virgin adult females were maintained at 25 °C and 50% r.h. as controls, while the remaining females were exposed to heat stress treatment. This involved a 2-h period of gradually increasing the environmental temperature from 25 up to 50 °C, followed by a 2-h period at 50 °C and finally a 2-h period of gradual reduction in environmental temperature from 50 back to 25 °C, the r.h. was maintained at 50% throughout. Fifty degrees was chosen as the heat stress temperature because preliminary studies with this culture had found that 55 °C caused mass mortality (JF Parkinson, unpubl.), and the aim was to test a sub-lethal treatment here. Virgin females were flash frozen in liquid nitrogen at 48 or 72 h after treatment and stored in absolute ethanol at −20 °C until use for qPCR analysis. A hundred second-instar juvenile mealybugs of mixed sex and 30 newly emerged adult male mealybugs were also exposed to the heat stress treatment. After treatment, the surviving individuals were counted.

Life-history study

Immediately following treatment, a subset of 40 adult virgin females from the treated group and 34 from the control group were separated out and mated with virgin males taken from the reared population. These females were exposed to two males each to ensure mating. The eggs laid by these females were counted, along with the offspring which then reached adulthood themselves under normal rearing conditions, and their sex ratio at adulthood was assessed.

Symbiont infection intensity study

We quantified the infection intensity of the two symbionts in heat stressed and control mealybugs using qPCR with the comparative C_T method and a host gene to control for DNA quantity (Schmittgen & Livak, 2008). qPCR primers and probes for the variable housekeeping 28S rDNA region – which is also used in studies for analysing the quantification of RNA in insects (Xue et al., 2010) – of the host *P. citri* (Gullan et al., 2003), and the 16S rDNA and 23S rDNA intergenic spacer region, a variable region previously targeted for qPCR by (Kono et al., 2008), of the γ-proteobacterial symbiont ‘*Ca.* Moranella endobia’ (Thao et al., 2002), were designed using the software Primer Express v.3.0 (Life Technologies, Foster City, CA, USA). Primers and probe for the *groEL* gene, a target previously used for qPCR of mealybug symbionts by (Kono et al., 2008), were developed for the *P. citri* strain of the β-proteobacterial symbiont, ‘*Ca.* Tremblaya princeps’ (Thao et al., 2002). These were designed using the software PRIMER3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and analysed using the software NetPrimer (Premier Biosoft International, Palo Alto, CA, USA) (Table 1). DNA was extracted from 25 individual adult mealybugs per treatment at 48 h after treatment, and 26 mealybugs at 72 h after treatment, by soaking each mealybug in distilled water before crushing in 100 μl of 10% Chelex and heating to 99 °C. The resulting product was centrifuged at 2 326 ***g*** for 20 min and the supernatant was pipetted off. Inhibitors from this supernatant were removed using the OneStep96™ PCR Inhibitor Removal Kit as per manufacturer’s instructions (Zymo Research, Irvine, CA, USA). DNA from individual mealybugs was diluted to 1/10 in molecular grade water for use in qPCR reactions. Triplett qPCR reactions for individual mealybugs were performed in a StepOnePlus™ Real-Time PCR System. Volumes of 10 μl were used for qPCR reactions with reagent final concentrations of 150 nM of each primer, 50 nM of probe, and 1× of ABI Taqman Universal Master Mix II with UNG (Life Technologies, Foster City, CA, USA). The cycle was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and the annealing temperature (collection step) for 1 min. An annealing temperature of 64 °C was used for *P. citri* and ‘*Ca.* Moranella endobia’ reactions and 60 °C for ‘*Ca.* Tremblaya princeps’ reactions. Mean concentrations of ‘*Ca.* Tremblaya princeps’ and ‘*Ca.* Moranella endobia’ were compared against the *P. citri* host control using the comparative C_T method to produce relative ΔC_T values. These were compared between control and treatment groups to produce $\Delta\Delta C_T$ values, which were used to calculate fold differences.

Statistical analysis

The numbers of eggs laid in the two treatments were tested for normality and homogeneity of variance, found to fit these assumptions, and then analysed using a General Linear Model. The percentages of surviving offspring and female offspring between treatments were analysed using a Generalized Linear Model with gamma distribution and log link function, using the likelihood ratio χ^2 test statistic. The numbers of females in each treatment which failed to oviposit were analysed using a Fisher’s Exact Test. qPCR data were processed using the comparative C_T method (Schmittgen & Livak, 2008), which calculates the relative density between target gene and host control gene. This was then converted to ratios between host control gene and target gene. Ratios were tested for normality and

Table 1 qPCR primers and probes used in the study for *Planococcus citri* host control, β-proteobacterial symbiont '*Candidatus* Tremblaya princeps', and γ-proteobacterial symbiont '*Candidatus* Moranella endobia'

Target organism	Target gene	Oligo name	Function	Fluorescence[1]	Oligo sequence 5′-3′	Product size (bp)
P. citri	28S rDNA (AY179451.1)	PcitriF	Forward primer	–	TCCGAGGAGACGTGTAAAAGTTC	56
		PcitriR	Reverse primer	–	CCTAGCCGCCGAAACGA	
		PcitriP	Probe	6FAM	ACGGCGCGTGTCGA	
'*Ca.* Tremblaya princeps'	*GroEL* (AF476091)	TprincepsF	Forward primer	–	TCCAAGGCTAAATACCCACA	155
		TprincepsR	Reverse primer	–	ATACAAAAGGTACGCCGTCA	
		TprincepsP	Probe	6FAM	CGCGCATACGAACAGTCGGA	
'*Ca.* Moranella endobia'	16S and 23S rDNA (AF476107.1)	MendobiaF	Forward primer	–	GAGCACCTGTTTTGCAAGCA	64
		MendobiaR	Reverse primer	–	CCCCTAGAGTTGTGGAGCTAAGC	
		MendobiaP	Probe	6FAM	AGTCAGCGGTTCGATC	

[1] 6FAM, 6-fluorescein amidite 5′ dye.

homogeneity of variance. The data were not found to fit these assumptions and were analysed using a Generalized Linear Model, again using a gamma distribution, log link function, and the likelihood ratio χ^2 test statistic. The C_T differences between control and treatment groups were converted into fold differences for display in Figure 2. All analyses were conducted in SPSS 20 (IBM-SPSS Statistics, Armonk, NY, USA).

Results

Life history

Both, second-instar mealybugs of mixed sex and adult male mealybugs experienced 100% mortality when exposed to the heat stress treatment. Two of 40 adult female mealybugs in the treated group died 1 h following the treatment. No further premature mortality was observed in this group, nor was any mortality observed in the 34 adult female mealybugs used in the control group. In the control group, two of 34 females failed to oviposit and one female produced an egg sac devoid of eggs. In the treated group, six of 38 surviving females failed to oviposit and one female produced an egg sac devoid of eggs (Figure 1). These females which did not lay eggs were discounted further from the experiment. Neither the proportion of females failing to lay eggs, the number of eggs laid, nor the brood survival (%) to adulthood differed significantly between treatment and control mealybugs (Fisher's Exact Test, P = 0.32; $F_{1,60} = 0.539$, P = 0.47; and $\chi^2 = 0.054$, d.f. = 60, P = 0.88, respectively), However, the sex ratio of offspring produced did differ significantly ($\chi^2 = 5.37$, d.f. = 60, P = 0.020), with treated females producing progeny with a more female-biased sex ratio at adulthood (Figure 1).

Symbiont infection intensity

Heat-stressed mealybugs had significantly reduced levels of '*Ca.* Moranella endobia' DNA relative to control mealybugs 48 h ($\chi^2 = 5.447$, d.f. = 49, P = 0.020) and 72 h following treatment ($\chi^2 = 11.332$, d.f. = 49, P = 0.001), with heat-stressed densities of '*Ca.* Moranella endobia' being reduced by 52% after 48 h and 50% after 72 h (Figure 2). Heat stress treatment was not found to cause a statistically significant difference in levels of '*Ca.* Tremblaya princeps' DNA 48 h following treatment ($\chi^2 = 2.71$, d.f. = 49, P = 0.10), although it did follow the same trend as '*Ca.* Moranella endobia', being reduced by 40%. However, levels of '*Ca.* Tremblaya princeps' DNA 72 h following treatment were significantly reduced ($\chi^2 = 8.338$, d.f. = 50, P = 0.004), with a 58% decrease (Figure 2). DNA densities for both symbionts were higher for both treatments after 72 h compared to 48 h, with control levels increasing 7.5-fold in '*Ca.* Moranella endobia' and 4.7-fold in '*Ca.* Tremblaya princeps'.

Discussion

The qPCR results showed that short-term heat stress at 50 °C led to reduced density of '*Ca.* Moranella endobia' and '*Ca.* Tremblaya princeps' DNA in *P. citri*. Absence of

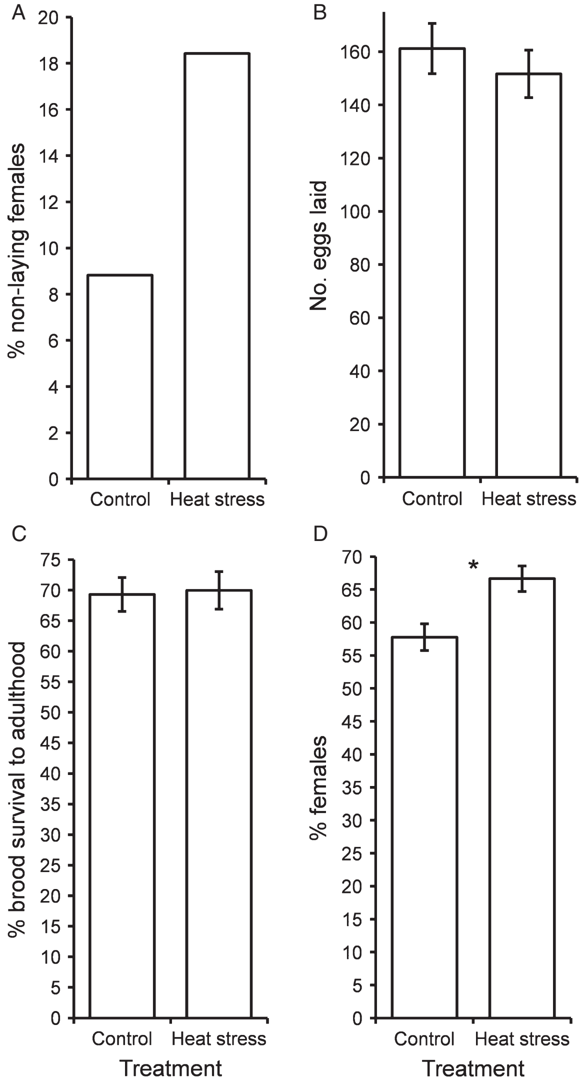

Figure 1 Mean (± SE) life-history parameters of adult female citrus mealybugs that were either exposed to short-term heat stress (50 °C) or control conditions. (A) Females (%) which failed to oviposit; (B) fecundity; (C) brood survivorship to adulthood; and (D) sex ratio of adult brood laid by treated females (% female prevalence). *$P<0.05$.

DNA indicates that the bacteria were digested or excreted by the host. This reflects a previous study, in which long-term heat stress at 39 °C physically damaged the symbiont system (Köhler & Schwartz, 1962). This may suggest that the heat is associated with cell death, or perhaps triggers an internal molecular mechanism or molecular cascade in *P. citri* which caused a host response to eradicate the symbiotic bacteria. However, in a previous study, the long-term heat treatment of 39 °C for 20 days resulted in the premature mortality of the adult mealybugs (Köhler & Schwartz, 1962), which was not observed for our short-term intense treatment. Decoupling of the symbiont reduction reflects the results observed in (Kono et al., 2008), with '*Ca.* Moranella endobia' reducing more rapidly. It may be that '*Ca.* Moranella endobia' is of lesser importance, or that '*Ca.* Tremblaya princeps' may digest or eject '*Ca.* Moranella endobia' before rupturing itself. The first suggestion is unlikely and the other appears maladaptive, as the biochemical dependency of these partners is obligate (McCutcheon & von Dohlen, 2011). Cell lysis has been suggested as a mechanism for the exportation of proteins from '*Ca.* Moranella endobia' to '*Ca.* Tremblaya princeps' (Husnik et al., 2013), and stressful conditions may disrupt this controlled event.

Although previous studies have found that constant rearing temperatures, varying typically across studies between 12 and 37 °C (Varndell & Godfray, 1996; Goldasteh et al., 2009; Ross et al., 2011), are greatly influential to the life-history parameters and survivorship of mealybugs, adult virgin female *P. citri* displayed strong physical resilience to the short-term intense heat stress treatment of 50 °C. This is despite this temperature killing 100% of second-instar and adult male mealybugs, and being only 5 °C less than the lethal temperature for adult females. Short-term heat stress did not impact the fecundity of the females, which suggests that key factors which determine the reproductive success of an individual occur during its development, and are only swayed by environmental temperature experienced in immature stages. As the symbionts are necessary for protein acquisition (McCutcheon & von Dohlen, 2011), they are probably most needed during the growth stages of the host, and are of lesser importance in adults, remaining present for transmission to the next generation. It would be of interest to know whether symbionts remain at reduced levels in treated virgin mealybugs for the remainder of their life span compared to control mealybugs or whether the offspring of females with reduced symbiont levels also have fewer symbiont cells. Adult male mealybugs naturally lose their symbionts post-pupation (Kono et al., 2008), so the loss of symbionts via heat stress is unlikely to be the cause of their mortality. Both adult males and juveniles are smaller than adult females and will have a larger surface area to volume ratio, thus likely rendering them more vulnerable to desiccation, which may explain their higher mortality rates.

Previous studies have shown that long-term exposure to raised temperatures during development can alter the sex ratio of mealybugs (Varndell & Godfray, 1996; Goldasteh et al., 2009; Ross et al., 2011). Our experiment has demonstrated that even a short transient exposure to higher

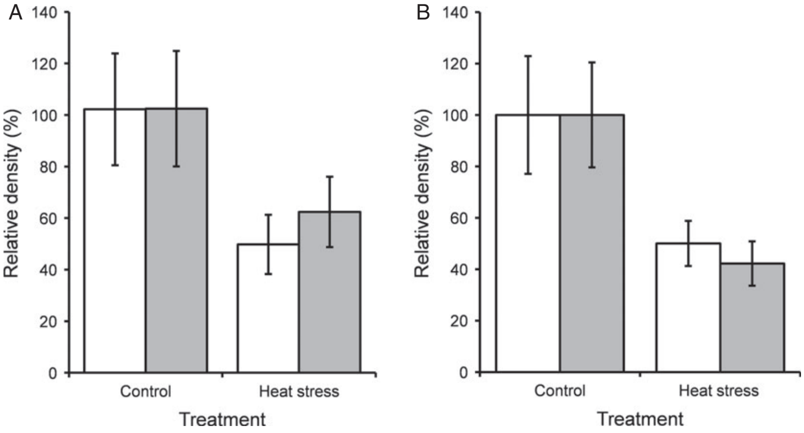

Figure 2 Mean (± SE) densities relative to host control gene of the '*Candidatus* Moranella endobia' (white) and '*Candidatus* Tremblaya princeps' (grey) endosymbionts in adult female citrus mealybugs that were either exposed to short-term heat stress (50 °C) or control conditions, at (A) 48 h following treatment (both n = 25), or (B) 72 h following treatment (n = 25 for heat stressed, n = 26 for control).

temperatures can cause an effect. Females from both the control and heat stress treatment produced brood with a female-biased sex ratio. However, the bias was slightly, but significantly, greater for treated females. This finding is in concordance with a previous study which found that hotter and more stressful conditions increased the prevalence of females in brood (Ross et al., 2011). Crowded females are more likely to produce male-biased brood, and age at mating is a complex interacting factor (Ross et al., 2010a). Mealybugs can facultatively adjust the sex ratio of their offspring through paternal genome elimination in males (Schrader, 1921; Brown & Nelson-Rees, 1961; Ross et al., 2010b, 2012), and is likely related to heterochromatic proteins (Buglia et al., 2009). The adult sexes are dimorphic, males being winged and dispersing and females being paedomorphic and sessile. There may be adaptive reasons for adjusting sex ratios following heat stress, or temperature may non-adaptively alter the determination mechanisms. Conversely, male brood of heat-stressed females may have suffered a higher mortality rate than those of non-heat-stressed females.

These results, along with the findings that symbiont density is reduced in post-reproductive females (Kono et al., 2008), indicate that host physical deterioration, perhaps triggered by senescence or stress, sways the relationship between host and bacteria. Although these symbionts are essential for the overall survival of the host, cost is incurred with maintaining a symbiont, and some environmental conditions may initiate a purge. Conversely, stressful conditions and physical deterioration may render the host incapable of housing symbionts and meeting their requirements. Symbiont degradation caused by heat stress and that caused by host senescence may not necessarily occur via the same mechanism and it would be interesting to investigate whether other environmental factors, such as food supply, cold exposure, and host plant species, can also alter the density of symbionts in mealybugs. This experiment provides only a snapshot of the dynamic relationship between mealybugs and their obligate symbionts, and it is possible that females could have recovered their symbionts after the qPCR measurements were taken. Such a recovery mechanism would imply that adult mealybugs are adapted to cope with symbiont fluctuation; hence, their reproductive fitness was unaffected. However, although fecundity was not affected, other fitness traits, such as immunocompetence or the ability to exploit different environments and host plants of other species, were not investigated in this study and may serve as significant factors when incorporated.

High temperatures have been tested in combination with other short-term disinfestation treatments, such as hot water immersion and ozone fumigation, as control strategies for mealybug pests on horticultural plants (Hansen et al., 1992; Lester et al., 1995; Hara et al., 1996; Dentener et al., 1997; Hollingsworth & Armstrong, 2005). Although often effective, high-heat treatments usually involve another element and may not be practical methods for some plants. Our results indicate that short-term, sub-lethal heat stress alone would not be an effective control strategy against mealybug infestations populated with many adults, although it would be highly effective against immature mealybug stages and does provide a potential experimental method for manipulating symbiont densities. It would be of great interest to observe whether other aspects of fitness were impacted, and whether other stressors also result in diminished symbiont densities.

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