Exploring the interactions of bacterial secondary symbionts (BSS) in wheat aphids, *Sitobion avenae* F. with parasitoids

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Abstract

Bacterial secondary symbionts (BSS) are renowned for their capacity to protect their hosts against natural enemies. *Sitobion avenae* (F.) is recently reported for *Hamiltonella defensa* and *Regiella insecticola* infections. Among natural enemies, parasitoids are the most effective bio-control agents. Host feeding strategy has been adopted by many parasitoids to increase their longevity and fecundity. Parasitoid-host interactions are highly specialized and depend upon the environmental factors and the genotypic backgrounds of the species involved. Besides the innate defence system, BSS composition of hosts may also alter the outcome of aphid-parasitoid interactions.

Genetically identical *S. avenae* clones possessed and lacked *H. defensa* and *R. insecticola* were used to assess host feeding and parasitism behaviour, host defence generality or specificity of these symbionts in this particular wheat aphid-symbiont system involving two parasitoids, *Aphelinus abdominalis* and *Aphidius rhopalosiphi*. *A. abdominalis* host feeding Reponses, towards of wheat aphids possessing and lacking BSS and genetic variations, studied at varying host densities and temperatures. In the last part of this study, *H. defensa* and *R. insecticola* were evaluated for defense against genetically different parasitoids *A. abdominalis* and *A. rhopalosiphi* for host feeding and parasitism.

BSS confer resistance to wheat aphids against *A. abdominalis* host feeding in both choice and no choice tests at all aphid host densities and temperatures tested. *A. abdominalis* host feeding response was positively correlated with host densities and temperatures up to a certain limit. *S. avenae* survivorship decreased significantly from lower to higher aphid densities and temperatures in choice and no choice tests. The most striking result was *A. abdominalis* host preference in feeding *S. avenae* without BSS in choice tests. Genetic or clonal preference in host feeding was also observed when *A. abdominalis* were exposed to genetically different *S. avenae* clones in all experiments. Wheat aphid-symbiont bond for its outcome was not affected by variable host densities and temperatures.

H. defensa conferred a general defence against both parasitoids but *R. insecticola* was significantly resistant against host feeding of *A. abdominalis* while it didn't provide defence to *S. avenae* against *A. rhopalosiphi*. *A. abdominalis* development in *S. avenae* was negatively affected by both of these bacterial symbionts but *A. rhopalosiphi* got negative impact only from *H. defensa*. Parasitoids were not involved in horizontal transmission of *H. defensa* and *R. insecticola* among *S. avenae* populations. These fitness advantages confer to their host aphids by these endosymbiotic bacteria expound the adaptive significance of their heritable and successful spread over many insect populations especially in aphids.

Chapter 1

General Introduction

Symbiosis, a purposeful interspecific association between two organisms, is omnipresent in the nature. Mostly, one of the partners is microbial organism referred to as symbiont. Symbioses vary in its dimensions of associations, role in host biology, partners' co-evolutionary history, and in genetic features of symbionts (Moran, 2006). Almost 15-20% of all insect species harbour symbiotic bacterial microorganisms. Generally, symbiotic bacteria are intracellularly reside in their host insect and are known as endosymbionts. They are grouped into primary and secondary symbionts depending upon their speciality. Primary symbionts are obligate for host survival and possess constant and highly specialized role in evolutionary life of host populations. They are facilitating their host insects to occupy a wide range of habitats through feeding flexibility (Douglas, 1998; Baumann, 2005; Moran, 2006).

Almost all aphid species harbour *Buchnera aphidicola* which is ranked among primary symbionts which reside in specialized cells called bacteriocytes (Douglas, 1998; Kikuchi, 2009). *Buchnera aphidicola* improves nutritional physiology in Hemiptera by compensating host aphids with deficit essential amino acids in their diet to increase host fitness and survival (Gündüz & Douglas, 2009). Many aphid species have adapted a wide range of symbiotic microorganisms in addition to their obligate bacterial associates known as bacterial secondary symbionts "BSS" (Moran et al., 2008; Ferrari et al., 2011). Bacterial symbionts are typically vertically transmitted from mother to offspring by manipulating the host reproduction system but inter- and intra-specific horizontal transmissions of BSS may occur at low rates (Moran & Dunbar, 2006; Chiel et al., 2009; Gehrer & Vorburger, 2012).

Aphids rely on symbiotic bacteria for their life activities like nutritional requirements (Gündüz & Douglas, 2009), thermal tolerance (Montllor et al., 2002), host protection from enemies (Haine, 2008), insecticide detoxification (Kikuchi et al., 2012), host plant adaptation (Tsuchida et al., 2002), body colouration (Tsuchida et al., 2010), sexual aberrations (Simon et al., 2011; Kageyama et al., 2012) and reproduction and development (Barribeau et al., 2010). The fitness advantages to the insect hosts and the dynamic transmission over host generations favour the wide spread of heritable microbial symbionts (Russell & Moran, 2005). In return, these bacterial endosymbionts manipulate the reproductive system of their hosts (White, 2011).

Hamiltonella defensa and *Regiella insecticola* are among the major BSS which occur with a frequency distribution of approximately 14-16% in aphid species and recently reported in *S. avenae* (Oliver et al., 2010; Łukasik et al., 2011). Both of them offer a wide variety of protection services to their insect hosts against their natural enemies including parasitic wasps, predators and fungal pathogens (Oliver et al., 2003; Piel et al., 2004; Łukasik et al., 2013). The protection mechanism adopted by *R. insecticola* is yet not clear but *H. defensa* rely on a toxin-encoding bacteriophage (APSE) for its virulence against parasitoids. These toxins appear to affect the parasitoids negatively (Oliver et al., 2009). Even parasitoid development may also be reduced by BSS after successful egg laying into the aphids (Nyabuga et al., 2010; Schmid et al., 2012) which may reduce parasitoid survival fitness. In addition to protection services, *R. insecticola* is also linked with host plant utilization of many aphid species. It is widely associated with pea aphid populations feeding on *Trifolium* plants (Tsuchida et al., 2002). *S. symbiotica* and *H. defensa* widen host fitness under high temperature (Montllor et al., 2002). Pea aphids, harbouring *S. symbiotica* and *H. defensa*, exceed their reproduction rate when they are confronted to alarm pheromones as an indicator of death risk (Barribeau et al., 2010). BSS can, thus, be regarded important evolutionary partners of their insect hosts (Feldhaar, 2011) through their journey of evolution.

The English grain aphid *Sitobion avenae* (F.) is a potential pest of cereal crops all over the word but especially in temperate climate (Alkhedir et al., 2010). It can establish and multiply tremendously in short time. It can cause direct and indirect damage to wheat crop which causes significant yield losses (Xu et al., 1998). Conventional breeding to develop resistant cultivars is important tool to cope with wheat aphids (Stoger et al., 1999) but about 5% of wheat crop is also chemically treated in European regions to avoid such loses (European Agricultural Statists, 2007). Aphids have the ability to reproduce very quickly and can establish insecticide resistance over time. So, it is hard to control aphids in the field (Foster et al., 2014). Above this fact, insecticides are also causing environmental pollution, changes in the ecological patterns and harmful effects to natural enemies (Bommarco et al., 2011). In twenty-first century, effective biological control opportunities are focused than ever to overcome such risks and sustainability in farming production (Bale et al., 2008). Biological control is controlling insect pests of economic importance by releasing their natural enemies instead of using insecticides. (Hufbauer, 2002). Among natural enemies, parasitoids are considered as the most effective agents in biological control because of their higher

reproduction, fast growth, easy rearing and positive response to increasing aphid population densities (Boivin et al., 2012; Yang et al., 2012).

Parasitoids are key player in almost all terrestrial ecosystems. They are diverse, plentiful and good ecological exploiter to influence the host population dynamics (Hassell, 2000; Godfray, 2007). They have intermediate life history between predators and parasites and possess an intrinsic capacity of responses to environmental factors (Godfray, 2007) and maintain natural balance of host populations in ecosystems (Rehman & Powell, 2010). In addition to parasitisation, many hymenopteran parasitoid species use their host as feed by consuming their host body materials. Parasitoid host feeding might be destructive to the host populations which causes considerable additional host mortality (Burger et al., 2004). Host feeding strategy has been adopted by parasitoids to increase their longevity and fecundity (Heimpel & Collier, 1996). Non-reproductive host feeding by parasitoids may also be considered to improve the efficiency of biological control (Byeon et al., 2009) in IPM.

Parasitoid-host interactions are highly specialized and antagonistic to exhibit the co-evolutionary relationships in nature. Host behavioural response, parasitoid development in hosts and environmental factors specify host ranges for parasitoids (Li et al., 2002; Kraaijeveld & Godfray, 2009). In nature, aphids harbouring bacterial secondary endosymbionts along with their associated natural enemies are exposed to environmental changes. Behavioural, physiological and biochemical systems related to aphid and parasitoid fitness are determined by environmental factors. Parasitoids are positively correlated with host densities and temperature up to a certain limit. Host densities are important factor for parasitoid behaviour to use the available resources efficiently but the whole system is temperature dependent (Kidd & Jervis, 1989; Thomas & Blanford, 2003). The population dynamics of natural enemies and their host species depend upon the temperature variations (Leather, 1993). The cost to thermal changes may increase and may become an important factor for mortality and reproduction by interacting directly or indirectly via natural enemies (Dill et al., 1990).

Host-parasitoids interactions are influenced by host population and environmental variations. It is important to predict the outcome of such interactions to improve the biological control strategies (Mostowy & Engelstädter, 2011). Population dynamics and temperature variations are important factors which can affect the life history traits of aphids, BSS and parasitoids. Extreme temperatures can be deleterious for the population dynamics of aphids and BSS within the host organisms (Montllor et al., 2002) which may decrease aphid's genetic and

symbiotic resistance towards parasitoids (Bensadia et al., 2006). Temperature can also affect the ability and mobility of parasitoids to exploit the host populations (Hance et al., 2006) and tolerance to environmental stresses enables the parasitoids to regulate their host populations (Thomson et al., 2010).

Parasitoids are quite able to detect the presence of BSS in their host aphids (Oliver et al., 2012) and they also behave differently towards clonal or genetic variations among their target aphid populations (Li et al., 2002, von Burg et al., 2008, Vorburger et al., 2009). Their hosts have developed strong defences under selection pressure and in turn parasitoids have to evolve counter-resistance system to exploit the host successfully (Li et al., 2002; Kraaijeveld & Godfray, 2009). Addition to the innate defence system, aphids have adopted symbiotic microorganisms to enhance their resistance against parasitoids (Kaltenpoth & Engl, 2014). Adoption, tolerance, maintenance, and transmission of bacterial secondary symbionts among aphid populations are important in defence strategies which are evolved over long time (Haine, 2008). Multiple parasitoid species, encountering single aphid host, can alter the host defence mechanisms (Nuismer and Thompson, 2006) depending upon the genotypic backgrounds of aphids, parasitoids and bacterial symbionts (Schmid et al., 2012).

The literature is piled up with aphid-bacterial symbiont-parasitoid interaction studies with limited scope on pea aphids and to some extent with bean aphids in relation to *Aphidius* species (Oliver et al., 2003, Vorburger et al., 2009, Nyabuga et al., 2010). A comprehensive view of mechanisms and influences of bacterial symbiont compositions on aphid-parasitoid interactions needs to be established for the maintenance and wide spread of bacterial endosymbiosis among aphids. Wheat aphid *Sitobion avenae* (F.), *Aphelinus abdominalis* (Dalman) and *Aphidius rhopalosiphi* are not well reported in this regard. *A. abdominalis* is a good biological control agent against many aphid species possessing host feeding behaviour (Schelt, 1994, Enkegaard et al., 2013) while *A. rhopalosiphi* is one of the most abundant parasitoid species of Aphidiinae in European cereal fields in northern parts of continent (Muratori et al., 2004).

To fill this literature gap, our study is dealing with the *A. abdominalis* host feeding behaviour with regard to bacterial secondary symbionts (*H. defensa* and *R. insecticola*) in *S. avenae* host (Alkhedir et al., 2013). Genetically identical wheat aphid clones possessing and lacking these BSS were obtained by applying antibiotics through micro injections to cure BSS from these aphid clones rather than by haemolymph transfer to avoid any genetic mixing. We also used

genetically different clones without BSS and also with different BSS to confirm the clonal resistance against host feeding.

The following questions were addressed in this study.

- Do *H. defensa* and *R. insecticola* confer resistance to *S. avenae* against *A. abdominalis* host feeding at variable host population densities and temperatures?
- Is host feeding behaviour of *A. abdominalis* affected by *H. defensa* and *R. insecticola*, host population density and temperature interactions?
- Does *A. abdominalis* show host feeding preference between wheat aphids with and without *H. defensa* and *R. insecticola* at variable host population densities and temperatures?
- Do host wheat aphid genetic variations affect *A. abdominalis* host feeding behaviour at variable population densities and temperatures?
- Are *H. defensa* and *R. insecticola* general or specific in resistance against genetically different parasitoids *A. abdominalis* and *A. rhopalosiphi* for host feeding and parasitism?
- Are *A. abdominalis* and *A. rhopalosiphi* involved in horizontal transmission of *H. defensa* and *R. insecticola* in *S. avenae*?
- Does *A. rhopalosiphi* show host preference for parasitism between wheat aphids with and without *H. defensa* and *R. insecticola*?
- Do host wheat aphid genetic variations affect *A. rhopalosiphi* parasitism behaviour?
- Do *H. defensa* and *R. insecticola* affect the development of *A. abdominalis* and *A. rhopalosiphi* in *S. avenae*?

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Chapter 2

Host feeding behaviour of *Aphelinus abdominalis* (Dalman) in response to bacterial secondary symbionts in *Sitobion avenae* (F.)

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Summary

1. *Aphelinus abdominalis* (Dalman) is a solitary parasitoid of many aphid species which also possess host feeding ability. *Sitobion avenae* (F.) is recently reported for bacterial secondary symbionts "BSS" *Hamiltonella defensa* and *Regiella insecticola*. BSS are renowned for their capacity to protect their hosts against natural enemies. Parasitoids are able to detect BSS infection and genetic variation of their host for parasitism.

2. Genetically identical wheat aphid clones harbouring BSS and free of BSS were developed by using antibiotics via micro-injection technique. Host feeding and host preference response of *A. abdominalis* to BSS was studied at varying host densities in no choice and choice tests by exposing them to developed clones *S. avenae*. Impact of BSS on parasitoid development in host wheat aphids was also determined.

3. BSS confer resistance to wheat aphids against *A. abdominalis* host killing in both choice and no choice tests at all aphid host densities tested. *A. abdominalis* host feeding response was positively correlated with host densities. *S. avenae* killing due to *A. abdominalis* host feeding increased significantly from lower to higher aphid densities in choice and no choice tests. *A. abdominalis* development in wheat aphids was negatively affected from BSS. BSS from *S. avenae* were not horizontally transmitted via *A. abdominalis*.

4. The most striking result was *A. abdominalis* host preference in killing *S. avenae* without BSS in choice tests. Genetic or clonal preference in host killing was also observed when *A. abdominalis* were exposed to genetically different *S. avenae* clones.

5. Based on our findings, we might conclude that BSS not only confer resistance to their hosts against parasitism but also against host feeding or killing strategy of *A. abdominalis* in wheat aphids. Parasitoid can still be negatively affected by BSS during development after parasitizing wheat aphids. In addition, BSS and genetic variations both can change the host preference behaviour of parasitoids.

Keywords: Host feeding, symbionts, *Aphelinus abdominalis*, *Sitobion avenae*, host densities

Introduction

Symbiosis is the purposeful interspecific association of two or more organisms. Almost 15-20% of all insect species harbour symbiotic microorganisms and are grouped into primary and secondary symbionts. The symbiotic microorganisms are facilitating the insects to occupy wide range of habitats through feeding flexibility (Dow 1986; Baumann 2005; Moran 2006). Almost all aphid species harbour *Buchnera aphidicola* which is ranked among the primary symbionts (Douglas 1998). *Buchnera aphidicola* improves the nutritional ecology of aphids by compensating them with the deficit amino acids in their diet to increase their survival (Gündüz and Douglas 2009). In addition to Primary symbionts, bacterial secondary symbionts "BSS" are also common among many aphid species (Moran *et al.,* 2008; Ferrari *et al.,* 2011). In aphids BSS are known for their ability to confer resistance to their host against biotic (natural enemies) and abiotic (temperature) stresses (Montllor *et al.,* 2002; Scarborough *et al.,* 2005, Oliver *et al.,* 2010, Himler *et al.,* 2011, Lukasik *et al.,* 2013). Host plant specialization and aphid body colour may also be affected by BSS (Tsuchida *et al.,* 2004 and 2010). In case of conferring partial resistance to parasitoid species, BSS may also reduce the growth of parasitoid larvae developing in the parasitized host and prolong the larval developmental time (Nyabuga *et al.,* 2010, Schmid *et al.,* 2012) which may reduce parasitoid survival success. BSS are typically vertically transmitted from the mother to the offspring by manipulating the host reproduction system (Chiel *et al.,* 2009) but inter- and intra-specific horizontal transmissions of BSS may occur at low rates (Moran *et al.,* 2006, Chiel *et al.,* 2009, Jaenike *et al.,* 2007, Gehrer & Vorburger 2012). BSS can thus be regarded important evolutionary partners of their insect hosts (Feldhaar, H. 2011, Ferrari and Vavre 2011) through their journey of evolution.

The English grain aphid *Sitobion avenae* (F.) is a potential pest of cereal crops all over the word but especially in temperate climate (Leather1993, Alkhedir *et al.,* 2010). It can establish and multiply tremendously in short time. It can cause direct and indirect damage to the crop (Blackman and Eastop, 2000). As a result, *S. avenae* causes significant yield losses in wheat crop (George and Gair, 1979; Xu *et al.,* 1998). About 5% of wheat crop is chemically treated in European regions to avoid such loses (European Agricultural Statists 2007). Conventional breeding for resistant cultivars is another tool to cope with wheat aphids (Stoger *et al.,* 1999). *Sitobion avenae* is recently reported for two Bacterial Secondary Symbionts "BSS" *Hamiltonella defensa* and *Regiella insecticola* (Lukasik *et al.,* 2011).

Parasitoid-host interactions play a pivotal role in trophic levels by exploiting and regulating a wide range of insect communities (Godfray 1994). In addition to parasitisation, the adults of many hymenopteran parasitoid species also use their insect host for host feeding (Heimpel and Collier, 1996, Ueno & Ueno 2007) which causes considerable additional host mortality (Byeon *et al.,* 2010, Enkegaard *et al.,* 2013). Host feeding by parasitoids enhances their longevity and fecundity (Giron *et al.,* 2004, Rivero and West 2005, Ueno and Ueno, 2007). Non-reproductive host killing by many parasitoid species may also be considered to improve the efficiency of biological control agent (Byeon *et al.,* 2009) in IPM. Feeding and killing by parasitoids is positively correlated with host densities but host killing proportion is oppositely linked with it (Kidd and Jervis, 1989). So, host densities are important factor for parasitoid behaviour to use the available resources efficiently. Parasitoids are not only able to detect the BSS infection status of their host aphids (Oliver *et al.,* 2012) but also they behave differently towards clonal or genetic variations among their target host populations of aphids (Ferrari *et al.,* 2001, Li. *et al.,* 2002, Von Burg *et al.,* 2008, Vorburger *et al.,* 2009).

Aphelinus abdominalis (Dalman) (Hymenoptera; Aphelinidae) is a good biological control agent against many aphid species and also possess host killing behaviour (Schelt 1994, Enkegaard *et al.,* 2013).

The impact of BSS in aphid-parasitoid-interactions has been investigated in detail in pea aphids and to some extent in bean aphids in relation to *Aphidius* species (Oliver *et al.,* 2003, Nyabuga *et al.,* 2010, Vorburger *et al.,* 2010). Most of the researchers focused on parasitism behaviour of parasitoids towards BSS. To the best of our knowledge we are not aware of papers dealing with the host feeding behaviour of parasitoids with regard to the BSS constitution of the aphid hosts and genetic variations in aphids at variable aphid densities. *Sitobion avenae* (F.) and *Aphelinus abdominalis* (Dalman) are not well reported in this regard. To fill this literature gap, we designed a study involving *Sitobion avenae*, its BSS (*Hamiltonella defensa*, and *Regiella insecticola*) and *Aphelinus abdominalis* interactions. Our study system was consisted of four genetically identical wheat aphid clones possessing different BSS. The antibiotics were applied to these aphid clones through micro injections to cure the BSS from these aphid clones to obtain genetically identical clones without BSS rather than haemolymph transfer to avoid any genetically mixing. The previous studies showed only clonal variations to parasitism without BSS. We also used genetically different clones without BSS and also with different BSS to confirm the clonal resistance against host feeding. Genetically identical aphid clones with and without BSS were more helpful to study

the host preference behaviour of *Aphelinus abdominalis* feeding. We disentangle the role of BSS and the genetic background with regard to the behaviour of *Aphelinus abdominalis*.

We addressed the following issues:

- Do BSSs impart resistance to *Sitobion avenae* against *Aphelinus abdominalis* host feeding? (No Choice Experiment)
- Does *Aphelinus abdominalis* show host preference for feeding on aphids with and without BSSs? (Choice Experiment)
- Is host feeding behaviour of *Aphelinus abdominalis* affected by BSS and host population density interactions?
- Do BSSs affect the development of *Aphelinus abdominalis* in *Sitobion avenae*?
- Is *Aphelinus abdominalis* involved in horizontal transmission of BSS in *Sitobion avenae*?

Materials and methods

Insect cultures

Sitobion avenae: Two clones (in the following named 5 and 7) of *Sitobion avenae* were used in this study, which were established from single asexual female aphids, collected in Giessen (Germany) from wheat plants (Alkhedir et al., 2010). The two clones have a different genotypic background and harbour either *Hamiltonella defensa* in clone 5 or *Regiella insecticola* in clone 7 as bacterial secondary symbionts (BSS) (Alkhedir *et al.,* 2013), respectively. These clones were continuously reared on the wheat plants, cultivar "Dekan" (KWS GmbH. Germany). The wheat plants were grown in 11cm diameter plastic pots containing a 2:1 mixture of soil and sand. The pots were covered with transparent ventilated plastic cylinders, which were 10 cm wide and 30 cm high. Aphids were transferred to newly grown wheat plants at 15 days interval., The aphid cultures were kept at 20±1 ˚C and 70±10% RH with a 16:8 (L:D) hour light period in climatic chambers (WB 750 KFL; Mytron Bio-Und Solartechnik GmbH. Germany). The wheat plants were irrigated on alternate days. The wheat aphid clones reproduced parthenogenetically provided these conditions.

Aphelinus abdominalis: The parasitoid species *Aphelinus abdominalis*, used in this study, were obtained from re-natur GmBH. Germany. The parasitoids were provided with 50% honey solution on arrival and the female parasitoids were used for experiments next day.

Elimination of BSS by antibiotic

Hamiltonella defensa harboured by the wheat aphid clone 5 was selectively eliminated by using an antibiotic treatment. A mixture of ampicillin, cefotaxime and gentamycin, each at a dose of 250 mg/ml, was applied through micro-injection using fine glass needles (Simon *et al.,* 2011). The antibiotic solutions were injected at the rate of 0.1-0.2 μl/ mg of body weight of the aphid specimens. For the removal of *Regiella insecticola* from clone 7, ampicillin was injected to the aphids in the same way at the rate of 1 μg/ mg of the body weight of aphid specimens (Koga *et al.,* 2003). CO_2 anaesthetized wheat aphids of the second nymphal stage were used for this treatment. These antibiotic treated wheat aphids were transferred to wheat plants individually and allowed to reproduce for 48 hours. The newly produced nymphs were defined as G1. From the G1, five nymphs were randomly chosen from each of the injected wheat aphid clones and reared on the wheat plants until they became adult and produced a sufficient number of G2 offspring. The G1 mothers were then subjected to diagnostic PCR for their BSS infection status. The G2 nymphs from BSS negative G1 mothers were chosen for further rearing only. The wheat aphids were subjected to diagnostic PCR to confirm the elimination of BSS until G8 (Koga *et al.,* 2007) being further used in our experiments. These treatments resulted in four genetically identical wheat aphid clones either harbouring *H. defensa* (referred to as +5 in the following), or devoid of this BSS (referred to as -5 in the following) and aphid clones harbouring *R. insecticola* (referred to as +7 in the following), or devoid of this BSS (referred to as -7 in the following).

Diagnostic PCR for BSS infections

DNA from the four aphid clones was extracted following the CTAB protocol of Chen *et al.,* (2010). The presence of *Hamiltonella defensa* and *Regiella insecticola* in the clones was determined using a diagnostic PCR with specific primers for amplifying the 16S rDNA gene fragments: HDFn [5-ATGAAGTCGCGAGACCAAA-3], HDRn [5-GCTTTCCCTCGCAGGTTC-3], RIFn [5-GAAGGCGGTAAGAGTAATATGC-3], and RIRn [5-CCCCGAAGGTTAAGCTACCTA-3] respectively. PCR conditions were: 94°C for 3 min followed by 30 cycles of 94°C-30 S; 60°C-40 S; 72°C-90S and final incubation at 72°C for 8 minutes. Reactions were carried in a 25 μl volume with one μl of the DNA template containing 0.32 μM of each primer, 2 mM MgCl2, 200 μM dNTP's, 1x "Bioline" buffer and 0.25 units of Taq DNA polymerase. PCR products were visualized on 1.7%

agarose gel using Ethidium bromide. To verify the identity of the PCR products, DNA from PCR products was purified from the gel after visualization and sent for direct sequencing to LGC Genomics GmbH, Germany. The resulting sequences were compared to known sequences using the BLAST algorithm in NCBI for confirmations of BSS.

Experiments

Host feeding behaviour of *A. abdominalis* in response to BSS (No Choice Tests)

Second and third instar nymphs of *S. avenae* clone +5, -5, +7, and -7, respectively, were exposed to *A. abdominalis* females at the rate of 6, 12, 18, 24 and 30 wheat aphid nymphs for 24 hours in separate 90 mm Petri dishes. The Petri dishes were provided with filter papers and washed rooted wheat seedlings covered with wet cotton. The experimental conditions were 20±1 °C and 70±10% RH with 16:8 hour light and dark period. The experiments were replicated 16 times for each treatment with 16 controls without *A. abdominalis* female parasitoids. The *A. abdominalis* females were removed after 24 hours and used for DNA extraction. The dead aphids were counted after 24 and 48 hours. Aphids still alive were allowed to feed on the wheat seedlings until mummifying. Hatching *A. abdominalis* were used for hind tibia length measurement and DNA extraction. Replicates in which the introduced parasitoids escaped or died were discarded from the analyses, and these treatments were set-up again for data collection and analyses.

Host preference behaviour of *A. abdominalis* in response to BSS (Choice Tests)

Second and third instar nymphs of *S. avenae* clones +5, -5, +7, and -7, respectively, were exposed to *A. abdominalis* females at the rate of 6, 12, 18, 24 and 30 nymphs for 24 hours in 90 mm Petri dishes in the same petri dish at 50:50 ratio. To differentiate between the BSS positive and negative wheat aphids, the hind tarsi of one leg of one of the clones were cut under a magnifying glass with sterilized blades. This was performed with BSS positive aphids in half replications and with BSS negative aphids in the other half replications. The Petri dishes were provided with filter papers and wash rooted wheat seedlings covered with wet cotton. The experimental conditions were 20±1 °C and 70±10% RH with 16:8 hour light and dark period. For this experiment we used 16 replications for each treatment with 16 controls without *A. abdominalis* females. The *A. abdominalis* females were removed after 24 hour and the dead aphids were counted after 24 and 48 hours.

Host preference behaviour of *A. abdominalis* in response to clonal variation (Choice Tests)

Second and third instar nymphs of *S. avenae* clones either +5 and +7 or -5and -7 were exposed to *A. abdominalis* females at the rate of 6, 12, 18, 24 and 30 nymphs for 24 hours in 90 mm Petri dishes combined at a ratio of 50:50. To differentiate between the aphids of clones +5 and +7, and -5 and -7 the tarsi of one hind leg of one of the clones were cut under a magnifying glass. This was performed with +5 clone aphids in half replications and with +7 clone aphids in the other half replications. The same method was used when using the BSS negative clones in the choice tests. Data collection was similar to the previous experiments.

Effect of BSS on *A. abdominalis* development:

To determine the impact of BSS on the larval development of *A. abdominalis* reared in wheat aphids with and without BSS, the length of hind tibia of each emerged offspring of *A. abdominalis* from mummified wheat aphids was measured in μm as a proxy for their total body size (Godfray 1994).The individuals from the no choice tests were used for measurement under a magnifying binocular (Stemi 2000-C, Carl Zeiss, Germany) with a predefined scale. These data were measured for at least 30 *A. abdominalis* individuals per mummified aphids of clones +5, -5, +7, and -7, respectively.

Transmission of BSS via *A. abdominalis*:

To check, whether the BSSs are transmitted vertically to the parasitoids hatching from BSS-harbouring aphids, total DNA was extracted by using the CTAB method from the *A. abdominalis* females recovered from the no choice experimental set up after 24 hours. The extracted DNA was used in a diagnostic PCR with the same protocol as it was used for wheat aphids to confirm BSS infection in *A. abdominalis*. The same procedure was also repeated for *A. abdominalis* specimens who emerged from aphid mummies in the no choice tests for BSS detection. The DNA was extracted from 30 *A. abdominalis* individuals collected from mummified wheat aphids of clones +5, -5, +7, and -7, respectively. 48 *A. abdominalis* individuals, removed from no choice tests of wheat aphid clones +5, -5, +7, and -7, respectively, after 24 hours, were used for DNA extraction.

Data analyses:

In no choice and choice experiments, the data of wheat aphids' mortality by *A. abdominalis* parasitoid host feeding was analysed using the factorial ANOVA (Statistix version 8.1

software) with the independent factors of host density and BSS. Differences among the means related to wheat aphid killing were compared with Fisher's Least Significant Difference (LSD) test at the significance level p=0.05. Data were square-root transformed [SQRT(x+0.5)], because host mortality in the controls compared to treatments did not meet the assumption of normal distribution. The graphs show the non-transformed data values (mean±SE) of aphids killed in the respective experiments.

For the determination of host preference behaviour of *A. abdominalis* host killing towards wheat aphids with and without BSS, percent mortality data were corrected by Abbott's formula (Abbott 1925) prior to the analyses. The difference of corrected mortality percentage (mean±SE) in aphids without and with BSS was used as an indicator of host preference in double choice test.

Formula = Corrected % mortality in aphids without BSS - Corrected % mortality in aphids harbouring BSS

The differences of corrected mortality percentage (mean±SE) of choice and no choice tests were compared with a paired t-test (Statistix version 8.1 software) at the 5% significance level for each of the aphids population densities individually.

 To determine the impact of BSS-harbouring aphid specimens on *A. abdominalis* adult fitness, the differences among the means of hind tibia length of *A. abdominalis* emerged from wheat aphids with and without BSS were compared with a paired t-test (Statistix version 8.1 software) at the 5% significance level.

Results

Host feeding behaviour of *A. abdominalis* in response to BSS (No Choice Tests)

No choice tests data, using clone 5 harbouring *Hamiltonella defensa* and clone 7 harbouring *Regiella insecticola*, show that BSS imparted resistance to *S. avenae* against host killing of *A. abdominalis* as compared to aphids without BSS (Fig. 1a & 1b). The negative impact of BSS on host killing of *A. abdominalis* was consistent at all aphid densities exposed to the parasitoid. Aphids killed by *A. abdominalis* host feeding varied significantly (p<0.005) when 6 to 18 aphids were offered per test. However, at higher aphid densities the aphid mortalities varied non-significantly when 18, 24 and 30 aphids were offered irrespective of BSS

presence or absence in wheat aphids. *A. abdominalis* host killing response towards different host densities was similar in both of the no choice tests. The lowest host killing was 1.25 and 2.12 aphids in clone 5 and 1.31 and 2.18 aphids in clone 7 respectively when 6 aphids with and without BSS were exposed to *A. abdominalis*. 7.31and 10.31 aphids in clone 5 and 7.12 and 10.37 aphids in clone 7 were killed when 30 aphids with and without BSS were offered to *A. abdominalis* respectively. The aphid mortality in controls was zero at 6, 12 and 18 aphids per test in both of the no choice tests. 0.125 and 0.25 aphids were dead at 24 aphids and 0.375 and 0.5 aphids were dead at 30 aphids per test in clone 5 while in clone 7, 0.25 aphids were dead at 24 aphids and 0.25 and 0.375 aphids were dead at 30 aphids in controls with and without BSS respectively. *A. abdominalis* host feeding capacity is positively correlated with host densities up to a plateau where it remains constant even more hosts are available for feeding and killing. These results also show that there is no possible effect of interaction between BSS and host densities on host killing behaviour of *A. abdominalis* in our no choice tests.

Host preference behaviour of *A. abdominalis* in response to BSS (Choice Tests)

The choice tests for clone 5 harbouring *Hamiltonella defensa* and clone 7 harbouring *Regiella insecticola* showed that BSS changed the aphid killing patterns by *A. abdominalis* as compared to no choice tests. The aphid mortalities increased significantly ($p<0.005$) in aphids without BSS (Fig. 2a & 2b) and reduced in aphids harbouring BSS (Table 1). The differences of corrected % mortalities in choice tests were more than twofold ($p<0.005$) of the differences of corrected % mortalities in no choice test at all the aphid densities for all of the wheat aphid clones (Table 1). The differences of corrected % mortalities were 14.58%, 17.7%, 15.99%, 15.94 and 9.84% in no choice test as compared to 41.67%, 33.33%, 30.56%, 25.85% and 18.69% in choice test at 6, 12, 18, 24 and 30 aphids in clone +5 and -5. The results were also similar for the clone +7 and -7. The higher difference of corrected % mortality by *A. abdominalis* host killing in choice test indicates host preference for wheat aphids without BSS when *A. abdominalis* has to choose between wheat aphids with and without BSS. *A. abdominalis* host killing increased significantly ($p<0.005$) with increase in aphid numbers offered from 6 to 30 aphids irrespective of BSS (Fig. 2a & 2b). Host killing and aphid densities are positively correlated. The lowest host killing was observed at 6 aphids and the highest host killing was seen at 30 aphids per test with and without BSS respectively.

The aphid mortality in controls was zero at 6, 12 and 18 aphids but 0.125 aphids were dead at 24 aphids and 0.25aphids were dead at 30 aphids in clone 5 while in clone 7, 0 and 0.125 aphids were dead at 24 aphids and 0.125 and 0.25 aphids were dead at 30 aphids in controls with and without BSS respectively. These results also show that there is no possible effect of interaction between BSS and host densities on host killing behaviour of *A. abdominalis* in our choice tests.

Host preference behaviour of *A. abdominalis* in response to clonal variation (Choice Tests)

We further studied the effect of genetic or clonal variation on host killing behaviour of *A. abdominalis* in another choice test. We offered wheat aphids from clone 5 and 7 together with different genetic background (Alkhedir *et al.,* 2013). One test was performed with aphids from clone +5 and +7and the other test with aphids from clones -5 and -7. The result showed that genetic or clonal variation also playing its role in the host killing behaviour of *A. abdominalis*. The aphids of clone 5 were significantly less fed and killed ($p<0.005$) by *A. abdominalis* host killing as compared to clone 7 irrespective of their BSS but this mortality was not significantly different when 6 aphids were offered for feeding (Fig. 3a & 3b). The aphids from clone -5 were also significantly less fed by *A. abdominalis* as compared to aphids of clone -7in the other choice test (Fig. 3a and 3b). *A. abdominalis* showed the same host killing behaviour towards different aphid densities irrespective of BSS (Fig. 3a & 3b) as it was observed in no choice tests. Host killing is positively correlated with aphid densities up to 18 aphids offered and then it becomes constant. The lowest host killing was observed at 6 aphids offered and the highest host killing was seen at 30 aphids with and without BSS respectively (Fig. 3a and 3b). The aphid mortality in controls was zero at 6, 12 and 18 aphids in both of the choice tests. 0 and 0.125 aphids were dead at 24 aphids and 0.25 and 0.125 aphids were dead at 30 aphids in clone 5 and 7 respectively. In the other test 0.125 aphids were dead when 24 aphids offered, in clone -5 and -7, respectively and 0.125 and 0.375 aphids were dead at 30 aphids in controls of -5 and -7, respectively. The clonal resistance of clone 5 and -5 against host feeding was consistent at all the aphid densities offered to *A. abdominalis* for feeding irrespective of the BSS presence.

Effect of BSS on *A. abdominalis* development

The hind tibia length measurements show that BSSs stressed the development of *A. abdominalis* in mummified wheat aphids among parasitized aphids and have significantly negative impact (p<0.005) on larval growth of *A. abdominalis* (Fig. 4). The hind tibiae of *A. abdominalis* were shorter when they reared in clone +5 and +7 as compared to clone -5 and -7, respectively. The highest hind tibia length was 369.92 μm in clone -7 verses 352.64 μm in clone +7 while these lengths were 367.36 μm and 353.28 μm in clone -5 and 5, respectively. *A. abdominalis* larval growth was higher in the wheat aphids without BSS as compared to aphid hosts harbouring BSSs.

Transmission of BSS via *A. abdominalis*

Diagnostic PCR for DNA extractions from *A. abdominalis*, removed from no choice tests after 24 hours, did not show any positive indication of BSS infection from wheat aphids. DNA extractions from *A. abdominalis* emerged from mummies of aphid clones +5, -5, +7, and -7, respectively, were also subjected to diagnostic PCR. The result was not different from the previous PCR result. It is obvious from our PCR results that *A. abdominalis* is not playing any role in horizontal transmission of BSS in *S. avenae* and *A. abdominalis*, emerged from aphids harbouring BSS, are also not infected by BSS.

Discussion

Host feeding behaviour of *A. abdominalis* in response to BSS

Aphids may possess certain bacterial secondary symbionts (BSS) and need to prove a positive contribution for the survival of their hosts for their own persistence and spread among the insect host community. Many BSS have been described for their ability to provide protection to their host insects against natural enemies i.e. parasitoids and pathogens (Oliver *et al.,* 2003&2005, Scarborough *et al.,* 2005, Vorburger *et al.,* 2009 & 2010, Łukasik *et al.,* 2013, Hansen *et al.,* 2012 and Schmid *et al.,* 2012). Łukasik *et al.,* reported first time *Hamiltonella defensa* imparted parasitoid resistance to wheat aphids in 2013. There should be more functional responses of these BSS in insect hosts for their existence and utilizing hosts' living system for their own benefits. Until now, all the studies told us about parasitism resistance of BSS in pea, wheat and bean aphids. BSS resistance against parasitoids host

feeding is not reported in literature to the best of our knowledge which is an important factor in biological control of aphids. Our results revealed that the resistant ability of BSS to benefit their host aphids against their natural enemies with regard to host feeding is also significant to increase the host survival at all aphid densities offered for feeding. As we could not find a single study about host feeding resistance of BSS, so, we can speculate that BSS imparted significant resistance to wheat aphids because there was no or negligible mortality in wheat aphids in control treatments. Another assumption might be possible that bacterial symbionts have helped to recover from host feeding injuries and hence increased their host survival. It is concluded that these bacterial symbionts proved another reason for their wide spread existence and spread in their insect hosts. This is also important because BSS may become a problem for biological control of pest aphids with the time if they provide resistance against all kinds of parasitoid and predator utility.

Host feeding behaviour of *A. abdominalis* in response to host densities

Parasitoids respond positively towards host densities for parasitisation and host feeding or killing (Fathipour *et al.,* 2006, Zamani *et al.,* 2006, Zang *et al.,* 2011, Hanan *et al.,* 2012, Tazerouni *et al.,* 2012, Yang *et al.,* 2012). These researchers showed positive correlation for host feeding and parasitism of different parasitoids and host densities of different aphid and whitefly species. Our data for *A. abdominalis* host feeding at different wheat aphid densities also showed the similar trend. Host mortalities were increased with increasing number of wheat aphids provided for feeding and killing. The host mortalities were not increased significantly at higher densities of wheat aphids as compared to lower aphid densities (Fig. 1a, 1b, 2a & 2b). This trend was also favoured by the previous studies mentioned above. These trends were clearly visible in both of wheat aphid clones 5 and 7 with and without BSS but there were always significantly fewer hosts killed in wheat aphids harbouring BSS as compared to wheat aphids without BSS ($p < 0.005$). The host density dependence maight be affected by the searching time and feeding capacity of the parasitoids. We could not find any study on host feeding behaviour with regard to BSS in insect hosts. We did not see any possible effect of BSS and host densities interactions on host feeding behaviour of *A. abdominalis*. The increasing host feeding trend was same at all the host densities with and without BSS and was always lower in aphid clones without BSS.

Host preference behaviour of *A. abdominalis* in response to BSS

BSS modified the host preference behaviour of *A. abdominalis* host feeding towards wheat aphids. This is not reported before in any study. The only study we could find was done by Cheng *et al.,* in 2011 with regard to primary symbiotic bacteria *Buchnera aphidicola*. He showed that *Aphis fabae* having *Buchnera aphidicola* were preferred by parasitoids. So, parasitoids can detect the infection status and decide their actions accordingly (Oliver *et al.,* 2012). In our choice tests, we have seen that *A. abdominalis* preferred wheat aphids without BSS over wheat aphids with BSS when came in touch with both types of wheat aphids. As wheat aphid clones are genetically identical but they are with and without BSS. We can say that BSS are quite able to modify the host preference behaviour of *A. abdominalis* host feeding.

Host preference behaviour of *A. abdominalis* in response to clonal variation

Genetic variations are innate source of host defenses. Wheat aphid clones +5 and -5 were more resistant against host feeding by *A. abdominalis* as compared to clones +7 and -7 with and without BSS in both of the choice tests. This variation in host feeding behaviour is associated with genetic variations of these aphid clones. Li. *et al.,* studied clonal resistance of pea aphid against *Aphidius ervi* in 2002 and showed that aphid clones can also resist against parasitoids. Clonal variations are able to influence defense phenomenon of *Myzus persicae* against *Aphidius colemani* and *Diaeretiella rapae* (Von Burg *et al.,* 2008). Similarly, clonal resistance in 34 pea aphid clones against two parasitoids and one fungal pathogen were due to genetic variations (Ferrari *et al.,* 2001). Genetic variations among 15 bean aphids clones were a defense source for another parasitoid (Vorburger *et al.,* 2009). They addressed that parasitoids exhibited clear variation for their resistance against parasitoids. So, it is predicted that the genetic variations can also modify the host feeding ability of parasitoids through behavioral or physiological defenses.

Effect of BSS on *A. abdominalis* development

 BSS reduce the growth of developing larvae of parasitoids in the infected aphids. Nyabuga *et al.,* 2010 studied the impact of *Hamiltonella defensa* and *Regiella insecticola* on the growth of *Aphidius ervi* in terms of their body mass. According to their results the BSS significantly reduce the mass of parasitoids together as compared to individually. Schmid *et al.,* also

reported in 2012 the similar negative effect of *Hamiltonella defensa* on the development of parasitoid in bean aphids. We evaluated the impact of BSS on larval development in wheat aphids in terms of hind tibia length measurement. Our data suggested that although parasitoids can survive in the presence of BSS but their fitness may reduce greatly. We have seen that the length of hind tibia was significantly decreased in aphids harbouring BSS as compared to wheat aphids free of BSS. It indicates that BSS can partially impart negative impact on parasitoid when they cannot resist them fully.

Transmission of BSS via *A. abdominalis*:

Vertical transmission of BSS is the thumb rule in insect host by manipulating the host reproduction system but horizontal transmission is also reported even at very low rate and rarely (Chiel*et al.,* 2009, Jaenike *et al.,* 2007, Gehrer and Vorburger 2012). Sometimes closely related BSS strains may occur in distant relative of the insect hosts (Russell *et al.,* 2003) which may be due to horizontal transmission of BSS. The potential routes for horizontal transmission of BSS in aphids can be Sexual transfer (Moran and Dunbar 2006), Transfer by parasitoids (Gehrer and Vorburger 2012) and Ingestion (Darby and Douglas 2003). Parasitoids cannot acquire BSS easily by host feeding or developing in the infected aphis hosts (Chiel *et al.,* 2009). This was also confirmed by our results of diagnostic PCR for parasitoids DNA extracted from *A. abdominalis* removed from experimental arena after 24 hours of exposure with BSS infected wheat aphids. The result was also same in case of *A. abdominalis* emerged from BSS infected wheat aphids hosts. This suggests that the parasitoids are not very common route of horizontal transmission of BSS among the insect communities because it would have a negative effect on their own survival and fitness.

Acknowledgements

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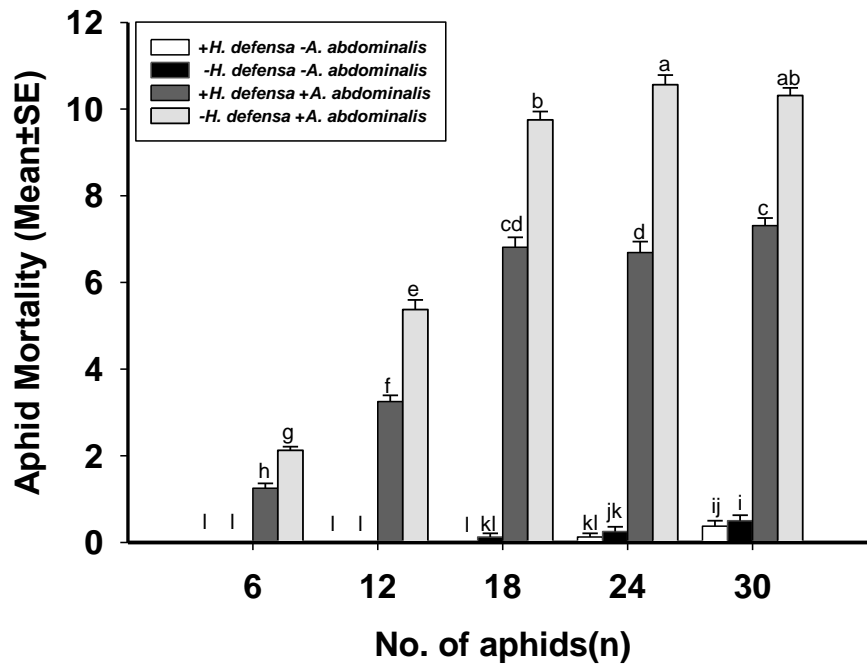*Figure 1a: A. abdominalis* host killing in S. avenae (with and without *Hamiltonella defensa*) at different host densities.

Figure 1b: A. abdominalis host killing of S. avenae (with and without *Regiella insecticola*) at different host densities.

Figure 2a: A. abdominalis host killing of S. avenae (with and without *Hamiltonella defensa*) at different host densities.
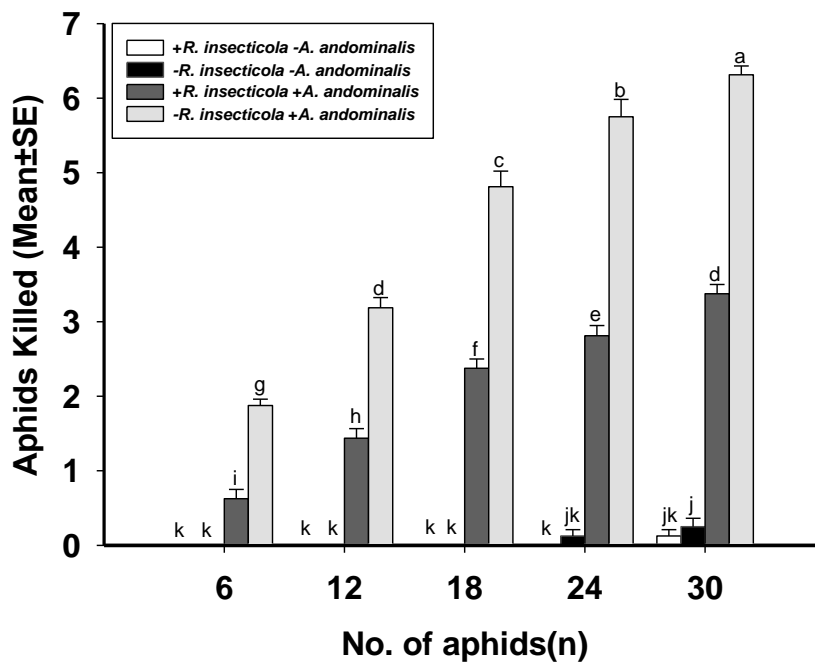
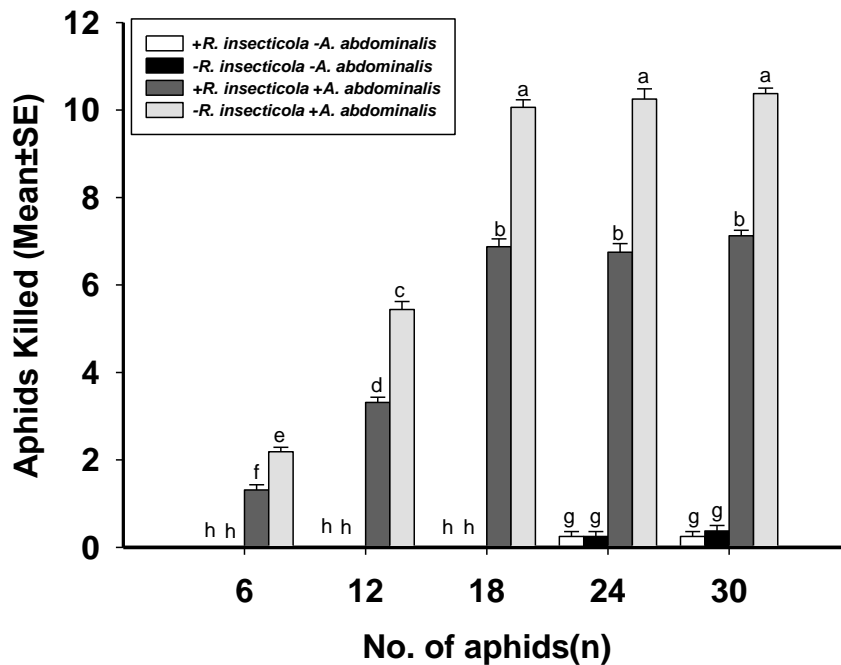*Figure 2b: A. abdominalis* host killing of S. avenae (with and without *Regiella insecticola*) at different host densities.

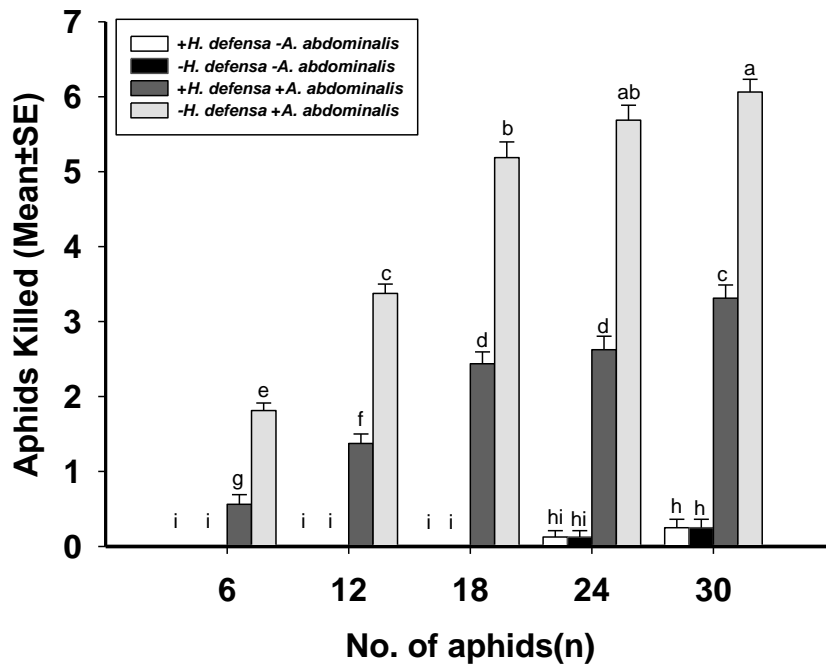*Figure 3a: A. abdominalis* host killing of *S. avenae* (with *H. defensa* and *R. insecticola*) at different host densities indicating clonal resistance.

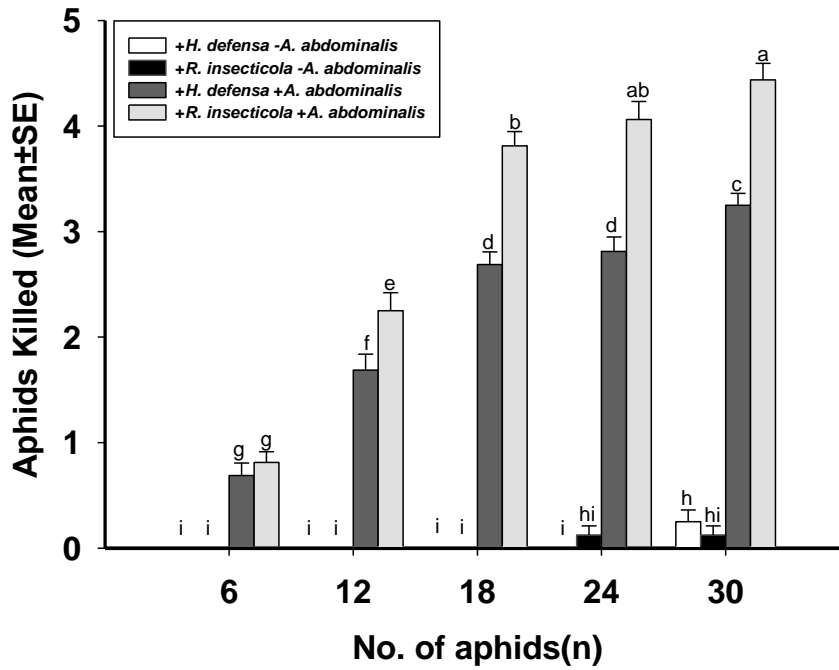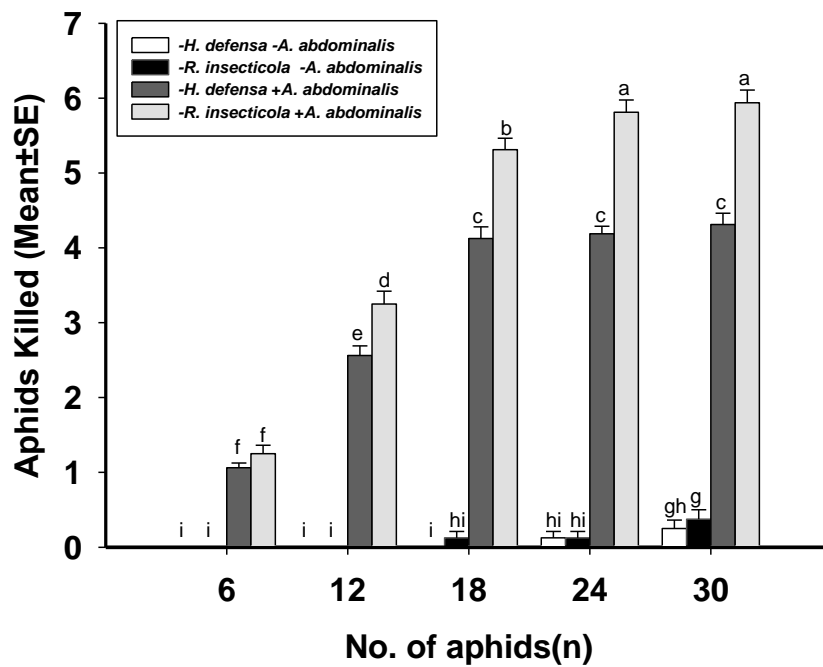*Figure 3b: A. abdominalis* host killing of *S. avenae* (without *H. defensa* and *R. insecticola*) at different host densities indicating clonal resistance.
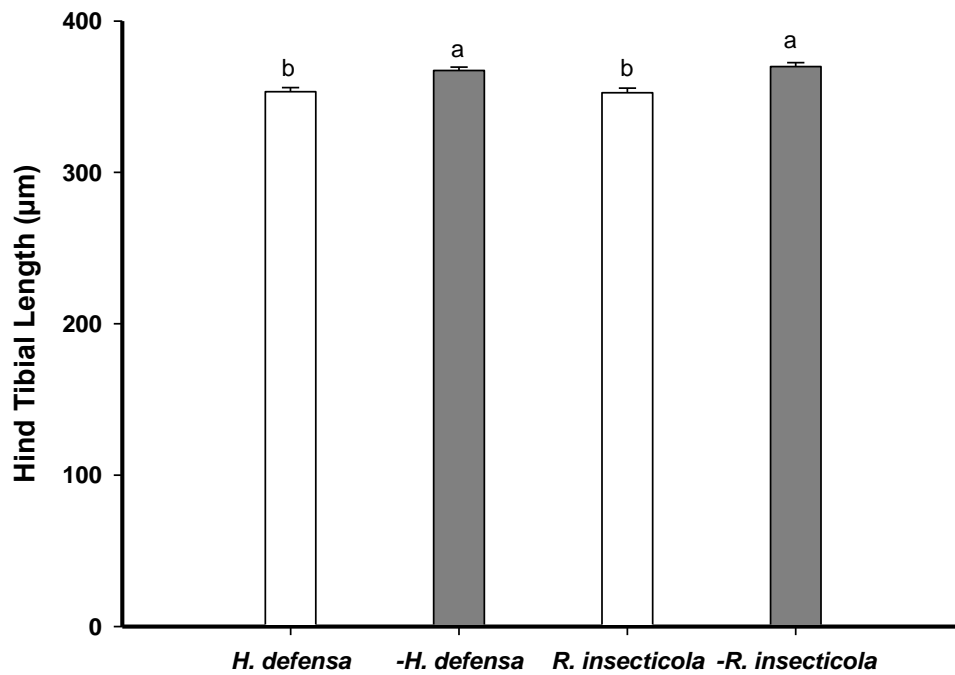
Figure 4: Hind tibia length of *A. abdominalis* developed in *S. avenae* (with and without *H. defensa* and *R. insecticola*).

Table 1: Differences of corrected % mortalities (Mean±SE) in *S. avenae* by *A. abdominalis* host feeding in no choice and choice tests.

	Clone 5		Clone 7	
Aphids	**No choice**	**Choice**	**No choice**	**Choice**
6	14.58±2.58b	41.67± 3.73a	14.58± 1.42b	41.67± 3.73a
12	17.70±1.68b	33.33± 3.04a	17.71± 1.68b	29.17± 3.23a
18	15.99±1.39b	30.56± 2.96a	17.71± 1.26b	27.08± 2.48a
24	15.94±1.45b	25.85± 2.49a	14.76± 139b	23.91± 2.60a
30	9.841±1.01b	18.69± 1.92a	10.64± 0.73b	19.26± 1.63a

Chapter 3

Impact of bacterial secondary symbionts on survivorship of *Sitobion avenae* (Fabricius) against *Aphelinus abdominalis* (Dalman) host feeding at variable temperatures

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ABSTRACT

Host-parasitoids interactions depend upon the climatic changes and the genotypes of their target hosts. Parasitoids are prone to climatic adversities for their fitness and survival as their feeding efficiency is temperature dependent. Parasitoids are ecologically important components in nearly all terrestrial ecosystems. The host feeding strategy has been adopted by many parasitoids to increase their longevity and fecundity. Almost all aphids possess extracellular bacterial secondary symbiont (BSS). They play a critical role in aphid's defensive system against their natural enemies and also help them to withstand environmental severities like temperature stress. We investigated the impact of bacterial secondary symbionts on the survivorship of *Sitobion avenae* against *Aphelinus abdominalis* host feeding at variable temperatures. Genetically identical wheat aphid clones, harbouring BSS and devoid of BSS, were used. BSS confer resistance to wheat aphids against *A. abdominalis* host feeding in both choice and no choice tests at all temperatures tested and hence increased host survivorship. *A. abdominalis* host feeding response was positively correlated with temperature gradients. Interestingly, *A. abdominalis* preferred to feed on *S. avenae* without BSS in choice tests. Clonal resistance also increased the survivorship of *S. avenae* when *A. abdominalis* were exposed to genetically different *S. avenae* clones. These fitness advantages conferred to their host aphids by endosymbiotic bacteria expound the adaptive significance of their heritable and successful spread over many insect populations, especially in aphids.

Keywords: survivorship, *Sitobion avenae*, *A. abdominalis*, Temperature, Host feeding

INTRODUCTION

Insect-microbe endosymbiotic interactions can be pathogenic or mutualistic. Mutualistic symbionts might be either primary (obligate) or secondary (facultative) depending upon their necessity (1). The insect populations are heterogeneous for such interactions (2). Aphids possess primary endosymbiotic intracellular bacteria, named *Buchnera aphidicola* to compensate essential amino acid deficiencies. They reside in specialized cells called bacteriocytes (3). Aphids also possess extracellular bacterial secondary symbionts (BSS) other than the primary symbionts. These BSS are not necessarily required by aphids for their survival but in mutual interactions of aphids and their endosymbiotic bacteria, aphids are reported to increase survivorship. Recent studies reveal that bacterial secondary endosymbionts are playing a critical role in aphid's defensive system against their natural enemies and help them to withstand environmental severities like temperature stress (4-6). The fitness advantages conferred to the hosts by these endosymbiotic bacteria make them successfully spread over host populations especially in aphids (7-9). The bacterial endosymbionts facilitate the host populations while passing through rapid evolution with inevitable ecological interactions (9). In return, these bacterial endosymbionts utilize the reproductive system of their hosts for their own maternal transmission (2).

Parasitoids are ecologically important components in nearly all terrestrial ecosystems. They possess an intrinsic capacity of responses to environmental factors (10) and are contributing to a biological control of host populations in ecosystems (11). Many of the parasitoid species use their host as feed by consuming their haemolymph. Host feeding might be destructive to the host organisms and can lead towards death of the hosts (12). This host feeding strategy has been adopted by parasitoids to increase their longevity and fecundity (13) and it also increases the effectiveness of parasitoids in biological control of insect pests (14).

In nature, aphids harbouring bacterial secondary endosymbionts along with their associated natural enemies are both exposed to environmental changes. Behavioural, physiological and biochemical systems are temperature dependent (15). Under temperature fluctuations, the cost to these changes may increase and may become an important factor for mortality and reproduction by interacting directly or indirectly via natural enemies (16). The population dynamics of natural enemies and their host species depend upon the temperature variations (17). The efficiency of natural enemies is largely affected by abiotic factors especially by temperature (18, 19) because of thermal sensitivity (20). Host-parasitoids interactions are

difficult to predict under environmental variations which may lead to problem for developing biological control strategies (21). Behavioural changes in aphids due to environmental factors can be important for their biological control (22) in agricultural pest management. Temperature variations are among the most important abiotic factors which are known to affect life history traits of aphids, BSS and parasitoids. They can affect longevity, fecundity, sex determination, size, development rate and seasonal occurrence of insects (23-25). Extreme temperatures can be deleterious for the population dynamics of BSS within the host organisms and in the context of global warming and climatic changes it is important to study the impact of thermal changes on the host aphid-parasitoids interactions as they are prone to climatic adversities which can cause lethal or sub lethal damages to them (4, 26). Aphid's genetic or clonal and bacterial resistance against parasitoids may decrease under thermal stress (27) but the parasitoids are more active at higher temperatures and they can also undergo diapause at low temperatures (28). The phenotypic plasticity and tolerance to environmental stresses enable them to exploit and regulate their host populations (29, 30).

Impact of temperature on host-parasitoid interactions had been studied by various researchers for better manipulation of parasitoids in biological control of aphids (27, 29, 31-34). Temperature affects the parasitism and parasitoids host feeding in a linear way until very high temperatures exert a negative impact on parasitoids (27, 35, 36). Increased average temperature potentially enhances the efficacy of biological control agents which is always a direct benefit to the parasitoids in parasitoid-host interactions as compared to aphids (22). There is need to match the climatic adaptation of the biological control agent with climatic conditions in the target region, is particularly relevant to classical introduction programs and to augmentative releases of commercially bred antagonists. Considerable progress is being made in our understanding of aphid-natural enemy interactions, leading to improved strategies for biological control, but practical and economic constraints still exist and need to be overcome, particularly in relation to mass-rearing and augmentative releases on outdoor crops (37).

To the best of our knowledge we are not aware of studies dealing with the parasitoids host feeding behaviour with regard to the bacterial secondary symbionts constitution of the aphid hosts and genetic variations in aphids at variable temperatures. We designed a study involving *S. avenae*, its BSS, *Hamiltonella defensa* (Moran) and *Regiella insecticola* (Moran), and *A. abdominalis* interactions at variable temperatures. In nearly all previous

studies, aphid clones with or without BSS have been compared, not taking into account the potential influence of the differences in the genetic structure of these clones.

Our study system was consisted of four wheat aphid clones possessing different BSS with an identical genetic background by artificially removing the BSS with antibiotics micro injections to avoid any genetic constraints. We also used genetically different clones without BSS and also with different BSS to confirm the clonal resistance against host feeding.

We addressed the following issues:

- Do BSSs affect the survivorship of *S. avenae* against *A. abdominalis* host feeding at increasing temperatures?
- Is host feeding behaviour of *A. abdominalis* affected by BSS and Temperature interactions?
- Does *A. abdominalis* show host preference for feeding on aphids with and without BSSs at variable temperatures?
- Do genetic variations affect the host feeding behaviour of *A. abdominalis* at variable temperatures?

MATERIALS AND METHODS

Insect Cultures

Sitobion avenae: *S. avenae* used in this study were collected from Giessen (Germany) on wheat plants. Two clones were established (referred as 5 and 7 in the following) from a single asexual female aphid. These two aphid clones harbour *H. defensa* in clone 5 and *R. insecticola* in clone 7 respectively as bacterial secondary symbionts (BSS) and have different genetic backgrounds (38). The wheat cultivar "Dekan" (KWS GmbH. Germany) was used to rear the aphid clones. The wheat plants were grown in soil and sand mixture (2:1) in 11cm diameter plastic pots covered with transparent ventilated plastic cylinders. The aphid cultures were kept at 20±1 ˚C and 70±10% RH with a 16:8 hour light and dark period in climatic chamber (WB 750 KFL; Mytron Bio-Und Solartechnik GmbH. Germany). The irrigation was done on alternate days. The wheat aphids reproduced parthenogenetically provided these conditions.

Aphelinus abdominalis: The parasitoid species *A. abdominalis*, used in these experiments, were obtained from re-natur GmBH. Germany. The parasitoids were fed on 50% honey solution on their arrival and the female parasitoids were used for experiments next day.

Elimination of BSS by antibiotics

The elimination of *H. defensa* harboured by the wheat aphid clone 5 was done by applying a mixture of ampicillin, cefotaxime and gentamycin (250 mg/ml each) through micro-injection using fine glass needles (39). The antibiotic solutions were injected at the rate of 0.1-0.2 μl/ mg of body weight of the aphid specimens. Ampicillin was injected to the clone 7 aphids in the same way at the rate of 1 μg/ mg of the body weight of aphid specimens for the removal of *R. insecticola* (40). The wheat aphids (second nymphal stage) were CO_2 anaesthetized for this treatment. The wheat aphids were shifted to wheat plants singly after treatment and allowed to reproduce for 48 hours. The newly produced nymphs were defined as G1. Five nymphs from the G1 were randomly selected to extract DNA by CTAB protocol (41) for the confirmation of the BSS by diagnostic PCR. The G2 nymphs from BSS negative G1 mothers were chosen for further rearing only. Elimination of BSS from clone 5 and 7 was confirmed by diagnostic PCR until G8 before the experiments. The diagnostic PCR was performed with specific primers to detect the presence of *H. defensa* and *R. insecticola* by amplifying the 16S rDNA gene fragments: HDFn [5-ATGAAGTCGCGAGACCAAA-3], HDRn [5-GCTTTCCCTCGCAGGTTC-3], RIFn [5-GAAGGCGGTAAGAGTAATATGC-3], and RIRn [5-CCCCGAAGGTTAAGCTACCTA-3] respectively. PCR conditions were: 94°C for 3 min followed by 30 cycles of 94°C-30S; 60°C-40S; 72°C-90S and final incubation at 72°C for 8 minutes. Reactions were carried in a 25 μl volume with one μl of the DNA template containing 0.32 μM of each primer, 2 mM MgCl2, 200 μM dNTPs, 1x "Bioline" buffer and 0.25 units of Taq DNA polymerase. PCR products were visualized on 1.7% agarose gel using Ethidium bromide. To identify the PCR products for BSS, DNA from PCR products was purified from the gel after visualization and sent for direct sequencing to LGC Genomics GmbH, Germany. The resulting sequences were then compared to the known sequences of *H. defensa* and *R. insecticola* using the BLAST algorithm in NCBI. Afterwards, we had clones 5 either harbouring *H. defensa* (referred to as +5 in the following), or devoid of this BSS (referred to as -5 in the following) and clone 7 harbouring *R. insecticola* (referred to as +7 in the following), or devoid of this BSS (referred to as -7 in the following).

Experiments

Survivorship of *S. avenae* against host feeding by *A. abdominalis* in response to BSS at variable temperatures (No Choice Tests)

10 nymphs of second and third instar *S. avenae* from clone +5, -5, +7, and -7 were exposed to *A. abdominalis* females at temperatures of 15, 20, 25, 30 and 35 °C, respectively, for 24 hours in 90 mm Petri dishes containing wheat seedlings with roots covered by wet cotton and filter papers in them. The Petri dishes were placed at 70±10% RH with 16:8 hour light and dark period. 16 replications were maintained over times for each treatment with 16 controls without *A. abdominalis* female parasitoids. The *A. abdominalis* females were removed after 24 hour and the dead aphids were counted after 24 and 48 hours. Replicates in which the introduced parasitoids escaped or died were discarded from the analyses, and these treatments were set-up again for data collection and analyses.

Host preference behaviour of *A. abdominalis* in response to BSS at variable temperatures (Choice Tests)

10 nymphs of second and third instar *S. avenae* from clone +5 and -5 were exposed to *A. abdominalis* females in the same Petri dish at temperatures of 15, 20, 25, 30 and 35 °C respectively for 24 hours at 50:50 ratios. The similar procedure was repeated for clone +7 and -7. To differentiate between the BSS positive and negative wheat aphids, the tarsi of one of the hind legs of the wheat aphids were cut under a magnifying glass with sterilized blades. This was performed with BSS positive aphids in half replications and with BSS negative aphids in the other half replications. *A. abdominalis* females were removed after 24 hour and wheat aphid clones +5, -5, +7 and -7 were separated by placing them into separate plates. The experimental conditions and data collection were the same as for no choice tests.

Host preference behaviour of *A. abdominalis* in response to clonal variations at variable temperatures (Choice Tests)

10 nymphs of second and third instar *S. avenae* from clone +5 and +7 were exposed to *A. abdominalis* females in the same Petri dish at temperatures of 15, 20, 25, 30 and 35 °C respectively for 24 hours at 50:50 ratios. To differentiate between the aphids from clone +5 and +7, the tarsi of one of the hind legs of the wheat aphids were cut under a magnifying glass with sterilized blades. This was performed with clone +5 wheat aphids in half replications and with clone +7 wheat aphids in the other half replications. The similar

procedure was repeated for clone -5 and -7. The experimental conditions, set-up and data collection were the same as for the above test.

Data analyses

In no choice and choice experiments, the data of wheat aphids' mortality by *A. abdominalis* parasitoid host feeding was analysed using a factorial ANOVA with the independent factors of host density and BSS. Differences among means related to aphid mortality were compared with Fisher's Least Significant Difference (LSD) test at the significance level of p=0.05. Data were square-root transformed [SQRT(x+0.5)], because host mortality in the controls compared to treatments did not meet the assumption of normal distribution. The graphs show the non-transformed data values (mean±SE) of aphids killed in the respective experiments.

For the determination of host preference behaviour of *A. abdominalis* host killing towards wheat aphids with and without BSS, percent mortality data were corrected by Abbott's formula prior to the analyses (42). The difference of corrected mortality percentage (mean±SE) in aphids without and with BSS was used as an indicator of host preference in double choice test.

Formula = Corrected % mortality in aphids without BSS - Corrected % mortality in aphids harbouring BSS

The differences of corrected mortality percentage (mean±SE) of choice and no choice tests were compared with a paired t-test at the 5% significance level for each of the aphid population densities individually. All analyses were performed in Statistix (version 8.1) software (43).

RESULTS

Survivorship of *S. avenae* against host feeding by *A. abdominalis* at variable temperatures in response to BSS

The survivorship pattern of wheat aphids, *S. avenae*, against host feeding behaviour of *Aphelinus abdominalis* was studied at variable temperatures in no choice tests. Bacterial secondary symbionts (BSS) significantly (p<0.005) increased the survivorship of wheat aphids from clones +5 (*H. defensa*) and +7 (*R. insecticola*) as compared to wheat aphids from clones -5 and -7 without BSS (Fig. 1a & 1b) against host feeding behaviour of *A.*

abdominalis. This positive impact of bacterial secondary symbionts on the survivorship of *S. avenae* against host feeding by *A. abdominalis* was consistent at all the temperatures except 15 °C when *A. Abdominalis* did not display host feeding behaviour. The pattern of wheat aphid's survivorship against *A. abdominalis* host feeding was similar in both of the no choice tests. The temperature exerted a negative effect on the survivorship of wheat aphids in all treatments. The survivorship of the wheat aphids decreased with an increase in temperature; a temperature 35 °C proved to be lethal for both the wheat aphids and as well as the parasitoids. The highest numbers of wheat aphids survived at 15 °C against host feeding by the parasitoid were as 9.5 and 9.56 aphids in clone +5 and -5 and 9.25 and 9.31 aphids in clone +7 and -7, respectively, while the lowest survivors were seen at 30 °C as 5.43 and 4.37 aphids in clone +5 and -5 and 6.31 and 4.93 aphids in clone +7 and -7 respectively. The highest temperature 35 °C caused 100% mortality of wheat aphids in all the treatments of both tests including the parasitoids. In controls, wheat aphid's survivorship was 100 % at 15 and 20 °C irrespective of BSS presence or absence. At 25 and 30 °C minimal wheat aphid's mortality was observed. *A. abdominalis* host feeding was positively correlated with temperatures up to 30 °C with their dependency upon the presence or absence of BSS in their host wheat aphids. The survivorship of wheat aphids against *A. abdominalis* host feeding was positively linked with the presence of BSS as we have observed an increased survivorship of wheat aphids possessing BSS also in control treatments although the difference was not significant. The survivorship of wheat aphids against *A. abdominalis* host feeding is negatively linked with the temperature. *A. abdominalis* fed on wheat aphids more actively with an increase in the temperature from 15 °C to 30 °C but it was always higher on wheat aphids without BSS.

Host preference behaviour of *A. abdominalis* in response to BSS at variable temperatures

Choice tests were performed to evaluate the host preference behaviour of *A. abdominalis* host feeding on wheat aphids when these parasitoids face aphids with and without BSS together. The choice tests for clones +5, -5, +7 and -7 showed that BSS changed the host feeding behaviour of *A. abdominalis* significantly (p<0.005) as compared to no choice tests (Fig. 2a & 2b). The differences of means for corrected % mortality of wheat aphids without and with BSS in no choice and choice tests were compared. The differences of corrected % mortality in choice tests were twofold of the differences of corrected % mortality in no choice test

(table 1) at all the variable temperatures for both of the aphid clones except at 15 °C. Because the parasitoids did not feed actively at 15 °C but in choice test, *A. abdominalis* have fed more on wheat aphids without BSS as compared to wheat aphids with BSS, showing their preference for feeding on wheat aphids without BSS. The higher difference of corrected % mortality by *A. abdominalis* host feeding in choice test indicated clear host preference behaviour when *A. abdominalis* had to choose between wheat aphids with and without BSS. *A. abdominalis* host feeding increased significantly ($p<0.005$) with increase in temperature from 15 °C to 30 °C irrespective of BSS (Fig. 2a & 2b) in all of the treatments of both choice tests. The survivorship of wheat aphids was also depending upon the BSS positively in choice tests and negatively depending upon the temperatures. Host feeding and temperature are positively correlated but 35 °C was lethal for both of wheat aphids and parasitoids when 100% mortality was achieved. The lowest host feeding was observed at 15 °C and the highest host feeding was seen at 30 °C in both of the choice tests with clones +5 and +7 respectively. The host feeding was always higher on wheat aphids without BSS at all the variable temperatures. The survivorship of wheat aphids in controls was 100 % at 15 and 20 °C irrespective of BSS presence or absence. At 25 and 30 °C minimal wheat aphid's mortality was observed both of the choice tests irrespective of BSS.

Host preference behaviour of *A. abdominalis* in response to clonal variation at variable temperatures

In other choice test, the genetic or clonal variation influenced the host feeding behaviour of *A. abdominalis* (Fig. 3a & 3b). Clone +5 was more resistant ($p<0.005$) to *A. abdominalis* host feeding as compared to clone +7, irrespective of their BSS (Fig. 3a). This genetically based resistance was consistent found at all the temperatures tested and was also found in choice tests using wheat aphids devoid of BSS. Clone -5 was also more resistant against *A. abdominalis* host feeding (Fig. 3b) as compared to clone -7 and was observed at all temperatures tested irrespective of BSS. Host feeding and temperature were positively correlated up to 30 °C; at 35 °C all wheat aphids and parasitoids were dead. The lowest host feeding was observed 15 °C and the highest host feeding was seen 30 °C respectively (Fig. 3a and 3b). The mortality in control treatments was zero at 15 and 20 °C but negligible mortalities were seen at 25 and 30 °C in both of the choice tests irrespective of BSS.

DISCUSSION

Survivorship of *S. avenae* against *A. abdominalis* host feeding in response to BSS at variable temperatures

The BSS, *H. defensa* and *R. insecticola*, expressed themselves in wheat aphid clones +5 and +7 by conferring significant partial resistance to their hosts against *A. abdominalis* host feeding behaviour as they have, already, been verified for host defence in pea aphids against parasitism (5). The symbiotic resistance, provided by both symbionts, was stable at all the temperatures tested. Unfortunately, there are no studies available which deal with the parasitoid host feeding interactions with aphids in relation to their bacterial endosymbionts at variable temperatures. We can only compare our results with parasitism studies. Cayetano and Vorburger studied the effects of heat shocks on the endosymbiotic resistance imparted to *Aphis fabae* (Scopoli) against *Lysiphlebus fabarum* (Marshall) parasitoid in 2013 (44). They reported that the ability of *H. defensa* to protect their hosts against parasitism was not affected by exposure to elevated temperatures. Likely, the ability of *H. defensa* and *R. insecticola* to protect wheat aphids against parasitoids host feeding was consistent even at elevated temperature regime. Thus, the relative fitness of bacterial endosymbionts, to protect their hosts, remains usually unaffected at elevated temperatures when encountered by parasitoids for host feeding or parasitisation. But at the same time, the host feeding efficiency of the parasitoids is positively linked with the temperature as their feeding and attack rates are increased with elevated temperatures up to a certain limit which shows their maximum capacity to feed on host aphids. Under certain conditions, this can help in the reduction of target insect pest populations (45). Our no choice lab trials revealed that the temperature changes can modify the host feeding capacity of the parasitoid *A. abdominalis* in his own favour linearly with temperature elevations until they suffered with mortality caused by lethal heat shocks to them. It might be a possibility that host feeding increased due to higher metabolism and activities. The daily host egg feeding of a female parasitoid, *Trichogramma turkestanica* (Meyer), against *Ephestia kuehniella* (Zeller) substantially increased with temperature gradients and reached to its maximum at 30 °C. Likely, the same temperature initiated the highest *A. abdominalis* host feeding on wheat aphids with the lowest host survival and vice versa at 15 °C (18). In another study, cotton aphids, *Aphis gossypii* (Glover), were offered to *Aphelinus asychis* (Walker) for host feeding at 18 and 30 °C. *A.*

asychis also killed more cotton aphids at 30 °C as compared to 18 °C (36). In case of parasitism, the parasitoid *Cephalonomia waterstoni* (Gahan) showed higher parasitisation rates on rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), with increasing temperatures; the highest parasitisation was recorded at 25 and 30 °C (46). Zamani et al., also showed that aphid parasitoids, *Aphidius colemani* (Viereck) and *Aphidius matricariae* (Haliday) attacking on the cotton aphids at five constant temperatures 10, 15, 20, 25, and 30 °C. 25 and 30 °C proved to be optimum temperature for the highest parasitisation (47). In 2002, Menon et al., reported a similar increasing trend of *Rhyzopertha dominica* (Fabricius) parasitisation by *Anisopteromalus calandrae* (Howard) with increase in temperature (48). Another parasitoid, *Praon volucre* (Haliday), also showed positive thermal dependency to parasitize *S. avenae* up to 25 °C; at temperatures above this level parasitisation rates declined (49). This might be due to the fact that parasitoids are more active feeders at elevated temperatures as compared to low temperature regimes and thermal dependency of parasitoids feeding and parasitisation is assumed from higher metabolism. On the other hand the foraging behaviour of another parasitoid, *A. matricariae*, was not influenced by the extreme temperature fluctuations (22). It is interesting that environmental fluctuations affect the aphis-parasitoid interactions but it depends upon the genotypes of the species involved also.

Effect of BSS and temperature interactions on *A. abdominalis* host feeding

No studies are available to explain the ability of bacterial endosymbionts to confer resistance to their hosts against parasitoid host feeding at variable temperatures or even under normal conditions. However, several studies published by Oliver *et al.,* (2003 & 2005), Scarborough *et al.,* (2005), Vorburger *et al.,* (2010), Łukasik *et al.,* (2013), Hansen *et al.,* (2012), and Schmid *et al.,* (2012), respectively, described bacterial endosymbionts for their ability to confer protection to their host aphids against different parasitoid species (50-56). This study would be first report to show more diversified function of BSS that they can also protect their hosts against host feeding parasitoids at elevated temperatures also. Predatory beetles such as *Adalia bipunctata* (Linnaeus) and *Hippodamia convergens* (Guérin-Méneville) killed more pea aphids harbouring *H. defensa* as compared to those who did not carry the symbiotic bacteria by host feeding. It could be an ecological cost of bacterial symbiont mediated resistance to pea aphids against parasitism (57, 58). The virulence of *Aphidius ervi* (Haliday) against pea aphids harbouring *H. defensa* was increased with temperature gradients thus decreased the survivorship of pea aphids at higher temperatures as temperature can be,

sometime, deleterious to the benefiting bacterial symbionts in aphids (27) depending upon the genotypes involved. Bacterial endosymbionts density can be reduced within individual host aphids to some extent but cannot be eliminated by thermal stress (4) but their ability to sustain host fecundity and to confer resistance to host aphids against parasitoids was not affected by the elevated temperatures (4, 59). Pea aphids infected with *H. defensa* became more susceptible to *A. ervi* attacks after thermal stress but pea aphids additionally harbouring PAXS secondary symbionts were able to maintain resistance against parasitism (60). As our findings are against to the above studies so it might be concluded that host-parasitoids interactions are also depends upon genotypic interactions which are able to change the feeding behavoir of natural enemies. Mainly parasitoids-protective endosymbionts interactions remain unchanged along the temperature gradients but survivorship of aphids harbouring bacterial symbionts may slightly reduce at elevated temperature (58). Moreover, bacterial secondary symbionts like *R. insecticola, H. defensa* and *Serratia symbiotica* (Moran) confer thermal tolerance to their host aphids to increase their fitness under heat stress and sustain their fecundity and longevity (61, 62) which enhance their survival., Low mortality of wheat aphids with BSS in control treatments also indicated that survival benefits were conferred to their host aphids as compared to wheat aphids without BSS although the difference was non-significant. This ability of thermal tolerance expounds the adaptive significance of heritable symbionts in the aphids with their interactions to abiotic environments.

Host preference behaviour of *A. abdominalis* in response to BSS at variable temperatures

Parasitoids can detect the presence of defensive bacterial endosymbionts in the aphids and modify their reactions accordingly to increase their fitness and better utilization of the hosts for their own favour (63). The primary symbiotic bacterium, *B. aphidicola*, was able to mould the host preference behaviour of *Lysiphlebus ambiguous* (Haliday). The parasitoids discriminated between symbiotic and aposymbiotic *A. fabae* and preferred symbiotic aphids harbouring *B. aphidicola* (64). It might be a reason that primary symbionts contribute nutritional deficiencies which make the aphids more suitable for parasitoid development. Host feeding behaviour of *A. abdominalis* was remoulded on exposure to wheat aphids with and without BSS. *A. abdominalis* preferred to feed on wheat aphids without BSS. This host feeding preference trend was observed along the temperature gradients. It is implicated from

the above discussion that the bacterial symbionts persist their ability to confer benefits their hosts under thermal stress as well and can also modify the feeding behaviour of parasitoids by changing the nutritional physiology of their hosts. It is also implicated that *A. abdominalis* is also a good opportunist, when encountered with both kind of wheat aphids with and without bacterial symbionts, to choose the least resistant ones.

Host preference behaviour of *A. abdominalis* in response to clonal variations at variable temperatures

Parasitoid virulence and host resistance to the parasitoids are ultimately depending upon the genotypes of the both species involved. Their interactions are modified by metabolic rates, cellular activities and temperatures (27). Genotypic and clonal variations among the aphid populations are known to confer resistance against parasitoids in addition to bacterial endosymbionts (50). With regard to host aphid-host feeding parasitoids interactions, the picture is not cleared. Clonal variations among pea aphid clones produce significant resistance *A. ervi* and *A. eadyi*. Aphid clonal resistance against parasitoid protected pink pea aphid clone from *A. ervi* as compared to green clone (65). *Myzus persicae* (Sulzer) showed that parasitism resistance against *A. colemani* and *Diaeretiella rapae* (McIntosh) is positively correlated to genetic or clonal variations among the host aphids (66). The black bean aphid, *A. fabae*, possesses genotypic variations in susceptibility to the parasitoid *L. fabarum* (67). Parasitism resistance was studied in 34 pea aphid clones against two parasitoids and it was assumed that genetic variations are significantly different in their resistance against the natural enemies (68). Likely, genetic variations may also be able to modify the host feeding behaviour of the parasitoids. Wheat aphid Clones +5 and +7 are genetically different. In both of the choice tests, taking individuals from clones +5/+7 together and individuals of clones -5/-7 together, the clone 5 was proved genetically resistant as compared to the clone +7 irrespective of BSS. Thus, genotypic variations are natural defense mechanism not only against parasitisation but also against host feeding parasitoids. It is also inferred that these natural defenses are stable enough to protect the aphids along temperature gradients as well. Pea aphid clones showed significant survival variations to parasitoid virulence but aphid clonal resistance was not affected significantly by slight temperature variations from 18 to 23 °C (69). In our experiments, Clone 5´s clonal resistance against *A. abdominalis* host feeding was also consistent with the temperature gradients. It is assumed that the physiological defenses against parasitoids are positively correlated with temperature gradients (70) but this

heritable clonal resistance of pea aphids to *A. ervi* at 20 °C was reduced when temperature was increased to 30 °C (27).

CONCLUSION

Environmental conditions matters in host-parasitoid interactions. Bacterial secondary symbionts have enough thermal stability to protect their hosts but it can be concluded that parasitoid virulence increases and host resistance decreases with temperature gradients and the natural enemies can be effective under specific abiotic stresses like environmental warming unless it is not harmful. Long exposure of such stresses can affect the efficiency of the natural enemies as well (71). Additionally, it might be assumed that the phenotypic effects of bacterial secondary symbionts as well as genetic variations modify the host feeding strategies of parasitoids. Finally, genotype x genotype x environment interactions should be addressed for effective biological control of aphids.

ACKNOWLEDGMENTS

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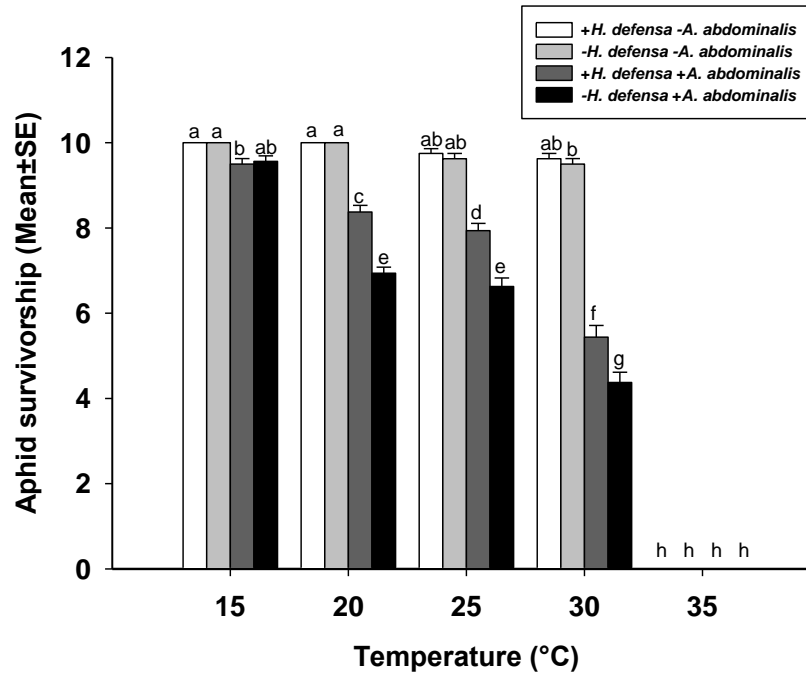
Figure 1a: Survivorship of *S. avenae* (with and without *Hamiltonella defensa*) against *A. abdominalis* host feeding at different temperatures.
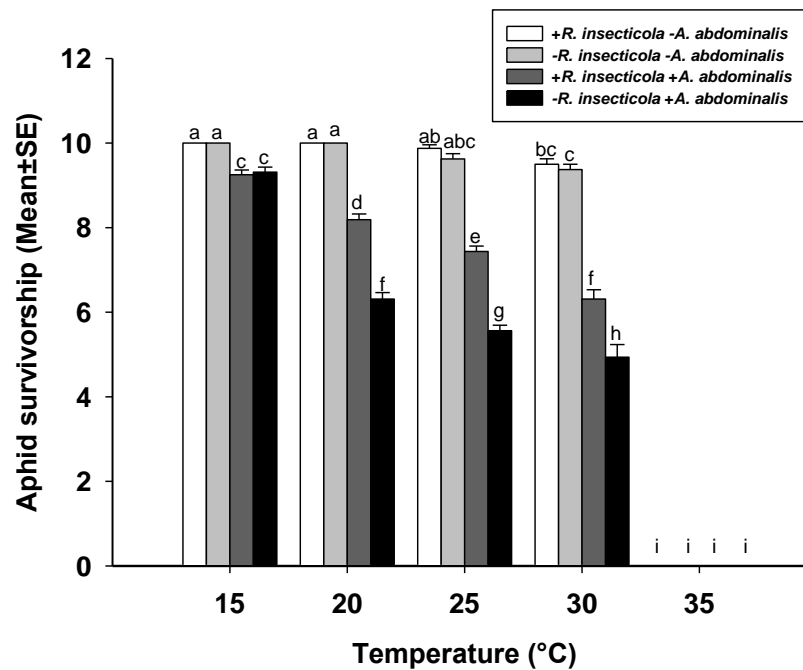

Figure 1b: Survivorship of *S. avenae* (with and without *Regiella insecticola*) against *A. abdominalis* host feeding at different temperatures.
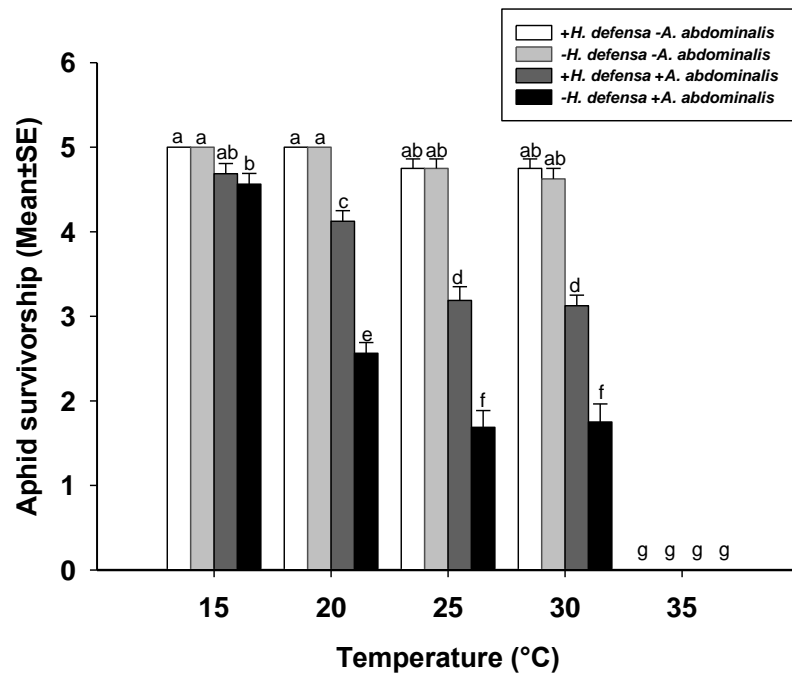
Figure 2a: Survivorship of *S. avenae* (with and without *Hamiltonella defensa*) against *A. abdominalis* host feeding at different temperatures.
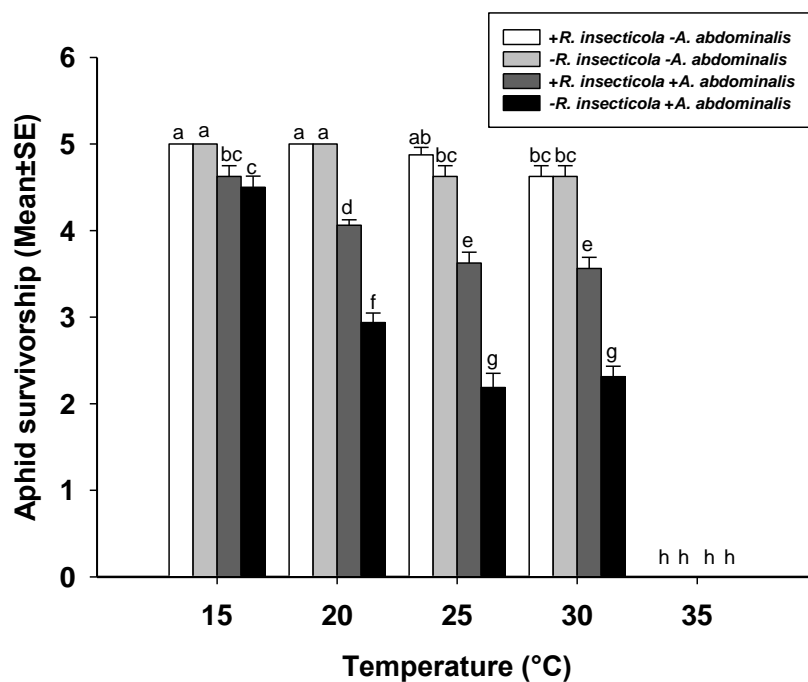

Figure 2b: Survivorship of *S. avenae* (with and without *Regiella insecticola*) against *A. abdominalis* host feeding at different temperatures.

Figure 3a: Survivorship of *S. avenae* against *A. abdominalis* host feeding (with *Hamiltonella defensa* and *Regiella insecticola*) at different temperatures indicating clonal resistance.
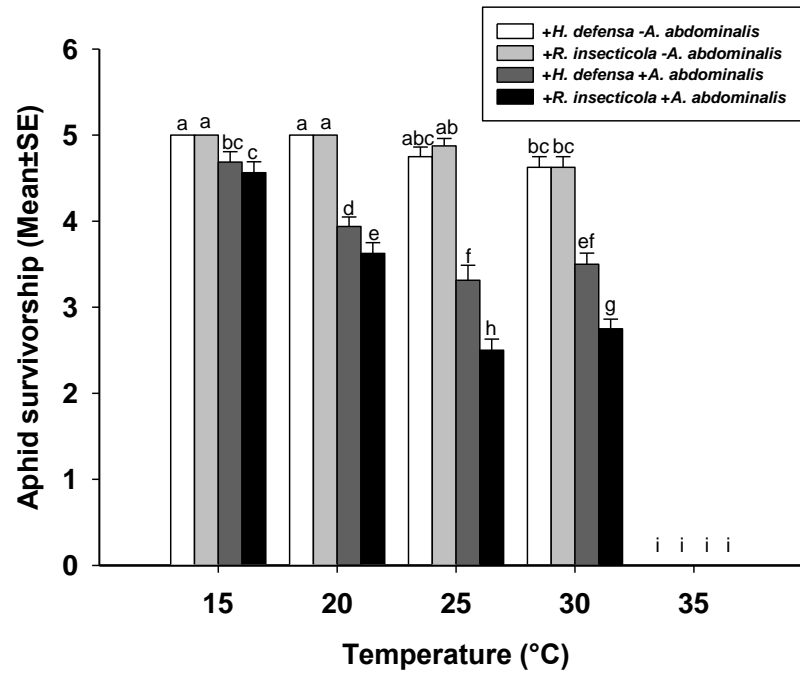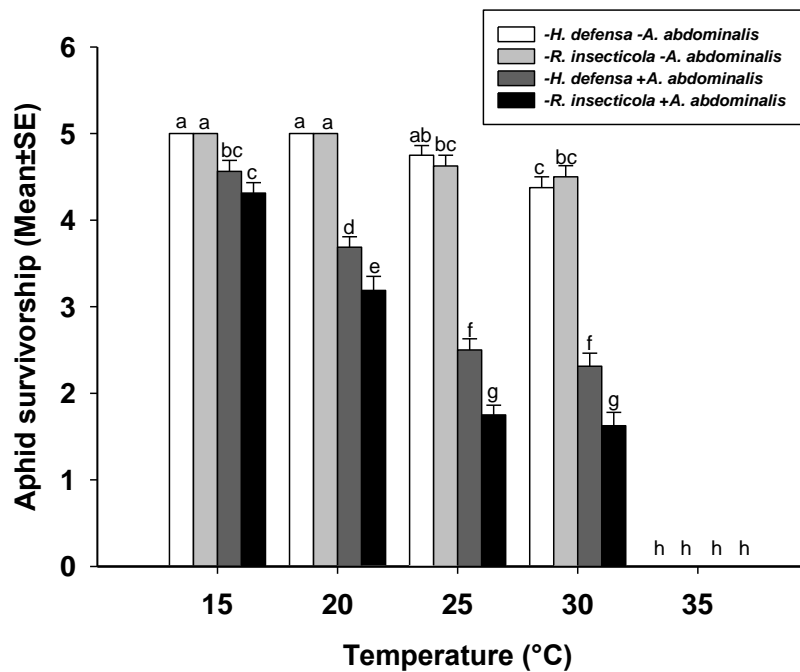

Figure 3b: Survivorship of *S. avenae* against *A. abdominalis* host feeding (without *Hamiltonella defensa* and *Regiella insecticola*) at different temperatures indicating clonal resistance.

Table 1: Differences of corrected % mortalities (Mean±SE) in *S. avenae* by *A. abdominalis* host feeding in no choice and choice tests at variable temperatures.

	Clone 5		Clone 7	
Temp	**No choice**	**Choice**	**No choice**	**Choice**
15°C	-0.62±1.92b	2.51±4.42a	0.62±1.71b	2.5±4.41a
20°C	14.37±1.57b	31.25± 4.06a	18.75± 1.79b	22.51± 2.51a
25°C	12.63±3.59b	32.51± 5.71a	17.43± 2.03b	27.52± 4.33a
30°C	10.34±4.22b	28.75± 4.59a	13.75± 4.2b	27.18± 3.62a
35°C	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a

Chapter 4

Specificity of bacterial secondary symbionts of *Sitobion avenae* in resistance against host feeding and parasitism

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Abstract

Parasitoid-host interactions are highly specialized. Host behavioural and physical defences determine host ranges for parasitoids. Genetic variations among aphid populations present substantial resistance to a particular parasitoid but particular host genetic background can exhibit different levels of resistance against multiple parasitoids. Besides the innate defence system, the symbiotic microorganisms can also significantly alter the outcome of such interactions. Symbiont-parasitoid specificity in resistance may modify aphid-parasitoid interactions. Two parasitoids, *Aphelinus abdominalis* and *Aphidius rhopalosiphi* and genetically identical *Sitobion avenae* clones possessed *Hamiltonella defensa* and *Regiella insecticola* and lacked them were used to assess defence generality or specificity of these symbionts in this particular aphid-symbiont system. The results revealed that *H. defensa* conferred a general defence against both parasitoids but *R. insecticola* was significantly resistant against host feeding of *A. abdominalis* while it didn't provide defence to *S. avenae* against *A. rhopalosiphi* in no choice trials. In choice trials, *A. abdominalis* host preference behaviour was modified by *H. defensa* and *R. insecticola* towards symbionts free *S. avenae* but these symbionts didn't alter host preference of *A. rhopalosiphi*. Genetic variations in *S. avenae* provided resistance against *A. abdominalis* but it could not alter *A. rhopalosiphi* virulence. *A. abdominalis* development in *S. avenae* was negatively affected by both of these symbionts but *A. rhopalosiphi* got negative impact only from *H. defensa*. It is inferred that bacterial endosymbionts may drive host selection process by parasitoids depending upon genotype-by-genotype specificity. Bacterial endosymbionts might be a critical factor to reshape the aphid-parasitoid ecological interactions.

Keywords: *Sitobion avenae*, host feeding, parasitism, *A. abdominalis*, *A. rhopalosiphi*

Introduction

Insect-microorganism symbiosis exists in nature with diverse interactions. Most of the insects in general, specifically aphids rely on symbiotic bacteria for their life activities like nutritional requirements [1,2], thermal tolerance [3], host defenses [4-7], insecticide detoxification [8], host plant adaptation [9,10], life history trait modifications [11,12], body colouration [13], male killing [14,15], sexual aberrations [16] and reproduction and development [17-20]. The fitness advantages to the insect hosts and the dynamic transmission over host generations favour the wide spread of heritable microbial symbionts [21,22]. Aphids have emerged as ideal organisms to study symbiotic interactions. For instance, *Buchnera aphidicola*, the primary bacterial symbiont in aphids, is associated with the provision of essential amino acid deficiencies in aphids' diet which improve the nutritional physiology in Hemiptera [2]. *B. aphidicola* is not the only bacterial symbiont harboured by aphid populations. The natural enemies are constant threat to the aphid's life. Despite natural clonal variations among aphid populations to resist the natural enemies [23], aphids require substantial investments in defense mechanisms for their survival. So, aphids have adapted a wide range of symbiotic microorganisms in addition to their obligate bacterial associates. *Hamiltonella defensa* and *Regiella insecticola* are among the major distribution [24]. They offer a wide variety of protection services to their insect hosts against their natural enemies including parasitic wasps, fungal pathogens and predators [4-7]. The protection mechanism adopted by *R. insecticola* is yet not clear but *H. defensa* rely on a toxin-encoding bacteriophage (APSE) for its virulence against parasitoids. These toxins appear to affect the parasitoids negatively [25]. In addition to protection services, *R. insecticola* is also linked with host plant utilization of many aphid species. It is widely associated with pea aphid populations feeding on *Trifolium* plants [9,10]. *S. symbiotica* and *H. defensa* confer higher fitness to pea aphids under high temperature [3]. Pea aphids, harbouring *S. symbiotica* and *H. defensa*, exceed their reproduction rate when they are confronted to alarm pheromones as an indicator of death risk [20]. Among natural enemies, hymenopteran parasitic wasps strongly curtail aphid populations in the ecosystem [26]. Parasitoids are key player in almost all terrestrial ecosystems. They are diverse, plentiful and good ecological exploiter to influence the host population dynamics [27,28]. They rely on successful parasitism in host for their existence as a part of their life cycle. Their hosts have developed certain defences to resist parasitoids attack under strong selection pressure and in turn parasitoids have to evolve counter-resistance system against these defences to exploit the host successfully [29,30].

Adoption, tolerance, maintenance, and transmission of bacterial secondary symbionts among aphid populations are important in defence strategies which are evolved over long time [31].

Parasitoid-host interactions are highly specialized and antagonistic interactions to exhibit the co-evolutionary relationships in nature [29,30]. Host behavioural response, parasitoid development in hosts and environmental factors specify host ranges for parasitoids. Host behavioural and physical defences determine recognition and orientation of parasitoids towards the host aphids. Once parasitoids overcome such defences and enter into the hosts, their virulence attributes directly interact with host immunity [32,33]. Besides the innate defence system, the symbiotic microorganisms can also significantly alter the outcome of such interactions [34]. Usually insect hosts are attacked by multiple parasitoids species. In return, hosts may evolve defences against these parasitoids that vary from general to specific. Many aphid species also face multiple parasitic wasp species and heritable bacterial endosymbionts confer resistance against some of these wasps [35]. Defence mechanisms are reshaped upon the recurrence of multiple parasitoids and host encounters and the rivalry of host and parasitoids can force the hosts to devise a general defence system against various parasitoids or to evolve a robust defence against a certain parasitoid and frail defence against other enemies [36-38].

Naturally, genetic or clonal variations among aphid populations present substantial differences to resist a particular parasitoid and a particular genetic background exhibits different levels of resistance against different parasitoid genotypes [23,39]. Particular bacterial symbiont in genetically identical host may or may not offer partial resistance against parasitism depending upon the genotypic backgrounds of the parasitoids indicating strong genotype x genotype specificity [40,41]. This symbiont-parasitoid specificity in resistance may modify aphid-parasitoid interactions which are, otherwise, not observed when defensive symbionts are not present in the aphid hosts [42]. Although there are ample evidences for genotype specificity in hosts-parasite interactions but the picture is less clear for generality of protection conferred by a particular symbiont in single host with identical genetic background against multiple species of parasitoid regarding host feeding and parasitism interactions. Whether *H. defensa* and *R. insecticola* in *Sitobion avenae* provide a general defence or they target only particular parasitoid species in this particular aphid-symbiont system? The answer can help to understand the driving forces promoting the wide occurrence of *H. defensa* and *R. insecticola* not only in *S. avenae* but also in many other aphid species. Genetically identical wheat aphid clones possessed *H. defensa* and *R. Insecticola* and lacked these symbionts were

developed by injecting antibiotics to these aphid clones through micro injections to cure these symbionts. Ultimately, we were capable to explore the specificity of particular isolates of *H. defensa* and *R. insecticola* in genetically identical *S. avenae* for their resistance against host feeding and parasitism by exposing them to genetically different parasitoids *Aphelinus abdominalis* and *Aphidius rhopalosiphi*. Their ability to affect the development of these parasitoids was also evaluated.

Materials and methods

Insects

Sitobion avenae: Two genotypes of *Sitobion avenae* naturally harbouring *Hamiltonella defensa* (clone 5) and *Regiella insecticola* (clone 7) collected from Giessen (Germany) were maintained on wheat plants as described already [43].

Aphelinus abdominalis: Re-natur GmbH. (Germany) provided the parasitoid *Aphelinus abdominalis*. The parasitoids were allowed to feed on 50% honey solution for 24 hours and female wasps were utilized next day for the host feeding trials.

Aphidius rhopalosiphi: Katz Biotech AG (Germany) provided the parasitoid *Aphidius rhopalosiphi* in the form of mummies. The mummies were stored at 24±1 ℃ for adult emergence. When the adults emerged, they were given 50% honey solution for 24 hours for feeding and mating and female wasps were utilized next day for the parasitism trials.

Elimination of BSS by antibiotics

H. defensa free clone 5 wheat aphids were obtained by injecting an antibiotic mixture of ampicillin, cefotaxime and gentamycin (250 µg/ml) through micro-injections with a dose of 0.1-0.2 µl/mg of aphid body weight [15]. Similarly, *R. insecticola* was cured from wheat aphids of clone 7 by injecting ampicillin (1 µg/ mg of the body weight of aphid) [44]. Antibiotic treatment was given to CO_2 anaesthetized wheat aphids. The treated wheat aphids were allowed to reproduce for 3-4 days on wheat plants. DNA was extracted from newly born nymphs following CTAB protocol [45] to verify the BSS removal by diagnostic PCR. HDFn [5-ATGAAGTCGCGAGACCAAA-3] and HDRn [5-GCTTTCCCTCGCAGGTTC-3] were specific primers used for *H. defensa* and RIFn [5-GAAGGCGGTAAGAGTAATATGC-3] and RIRn [5-CCCCGAAGGTTAAGCTACCTA-3] for *R. insecticola*. PCR conditions were:

94°C for 3 minutes followed by 30 cycles of 94°C-30S; 60°C-40S; 72°C-90S. Finally products were incubated for 8 minutes at 72°C. PCR reaction mixture was comprised of 1 µl DNA template, 0.32 µM of each primer, 2 mM $MgCl_2$, 200 µM dNTPs, 1x PCR buffer and 0.25 units of Taq DNA polymerase in 25 µl total volumes. 1.7% agarose gel was used to visualize PCR products using ethidium bromide. Eventually, we had wheat aphid clones 5 either possessed *H. defensa* (referred to as clone +5) or lacked *H. defensa* (referred to as clone -5) and 7 possessed *R. insecticola* (referred to as clone +7) or lacked *R. insecticola* (referred to as clone -7). BSS free wheat aphids were further maintained until 8th generation to stabilize the curing of BSS before trials and diagnostic PCR was used to verify the BSS removal at each generation.

Experiments

Response of bacterial secondary symbionts in *Sitobion avenae* against host feeding and parasitism (no choice trials)

A. abdominalis and *A. rhopalosiphi* females were released in 90 mm Petri dishes containing 12 wheat aphids (second and third stage) from clone +5, -5, +7, and -7 each. The Petri dishes had filter papers and rooted wheat seedlings covered by wet cotton. The trial was conducted at 20 ± 1°C and 70 ± 10% RH with 16:8 photoperiod. The controls were also maintained without parasitoids. After 24 hours, parasitoids were removed and the dead aphids were counted after 24 and 48 hours for *A. abdominalis* host feeding and control treatments. While dead aphids were also counted for *A. rhopalosiphi* treatments and its controls for 5 days as unknown or natural mortality. The aphid mummies were collected on 6th and 7th day for *A. rhopalosiphi* treatments and total mortality was comprised of dead and mummified aphids. Replicates in which the introduced parasitoids escaped or died were discarded and set-up again for data collection.

Host preference behaviour of parasitoids in response to BSS (choice trials)

12 wheat aphids from clone +5 and -5 (6 aphids each) were released in the same Petri dish along with the parasitoid females. Tarsi of hind legs of the wheat aphids were trimmed by using sterilized blades under a magnifying glass to discriminate the wheat aphids possessed BSS and lacked BSS. The trimming of tarsi was done for BSS positive aphids in half of the replications and vice versa. Similarly, choice trial was conducted for cone +7 and -7. The experimental conditions and data recording were the same as for no choice tests. The only

difference was that wheat aphids possessed BSS and lacked BSS were separated after 24 hours and put into the separate Petri dishes.

Host preference behaviour of parasitoids in response to clonal variation (choice trials)

12 wheat aphids from genetically different [43] clones +5 and +7 (6 aphids each) were released in the same Petri dish along with the parasitoid females. The trimming of tarsi was done for the wheat aphids from clone +5 in half of the replications and for the wheat aphids from clone +7 in rest of replications. Similarly, another choice trial was also conducted for clones -5 and -7. The experimental conditions and data recording were the same as for the above choice trials.

Impact of BSS on larval development of parasitoids

The lengths of hind tibia of emerged parasitoids were measured to assess the impact of BSS on larval development of parasitoids in aphids with and without BSS as an index of their body size [27] from mummified aphids (no choice trials) under a magnifying binocular (Stemi 2000-C, Carl Zeiss, Göttingen, Germany) with a predefined scale.

Horizontal transmission of BSS via parasitoids

Total DNA was extracted from all parasitoid females removed after exposure to wheat aphids and from 36 adult parasitoids emerged from wheat aphid mummies in no choice trials for BSS detection for both parasitoids species. Diagnostic PCR was performed in the same way as it was done for the wheat aphids to confirm BSS infection or contamination in the parasitoids.

Data analyses

All experiments were held with 16 replications over time. Factorial ANOVA (5% significance level) was performed to analyse mortality in wheat aphids caused by the parasitoids with BSS and parasitoids as independent and mortality as dependent factors. Fisher's Least Significant Difference (LSD) test was applied to compare differences among % mortality means. The data was square-root transformed [SQRT(x+0.5)] for statistical analyses because mortalities in the controls were not normally distributed as compared to treatments. All graphic figures show mean values (±SE) of % mortality in wheat aphids.

The ratios of corrected % mortality means [46] in wheat aphids harboured BSS and lacked BSS in choice trials were compared by paired T-test at 5% significance level to no choice

trials as an indicator of host preference behaviour in choice test. Ratios of corrected % mortality was obtained by dividing corrected % mortality in aphids without BSS by corrected % mortality in aphids with BSS.

Parasitoid fitness in response to BSS presence or absence was evaluated by comparing the % increase in the tibia of hind legs of the adults emerged from wheat aphids with and without BSS by running Fisher's Least Significant Difference (LSD) test after one-way ANOVA at 5% significance level. The means (±SE) of % increase in the tibia are presented by plot box. All statistical analyses were performed by using software Statistix version 8.1.

Results

Response of bacterial secondary symbionts in *Sitobion avenae* against host feeding and parasitism

Wheat aphid sublines of clone 5 represent significant differences in susceptibility to both of the parasitoids. Clonal line +5 harbouring *H. defensa* showed significantly (p<0.005) less mortality against host feeding by *A. abdominalis* and parasitism by *A. rhopalosiphi* as compared to clonal line -5. The partial resistance imparted by of *H. defensa* was consistent against both of the parasitoids and the mortality was decreased by 18% in wheat aphids harbouring *H. defensa* (Fig. 1a). Clonal lines of clone 7 showed significant (p<0.005) differences in susceptibility to host feeding by *A. abdominalis* but mortalities caused by *A. rhopalosiphi* parasitism didn't differ significantly (p>0.005). Clone +7 harbouring *R. insecticola* was more resistant against *A. abdominalis* as their mortality was lessen by ca. 18% as compared to clone -7 while the mortality was only 3% less in Clone +7 when they are exposed to *A. rhopalosiphi* which was not significant (Fig. 1b). The mortalities in all of the treatments were significantly higher than fatalities in control treatments. All control treatments showed zero mortality against host feeding by *A. abdominalis* while parasitism by *A. rhopalosiphi* caused nadir mortalities in controls (Fig. 1a & 1b).

Host preference behaviour of parasitoids in response to BSS

The choice trials for wheat aphids from clone 5 (harbouring and lacking *H. defensa*) and clone 7 (harbouring and lacking *R. insecticola*) showed that BSS modified the host feeding pattern of *A. abdominalis* as compared to no choice trials. Wheat aphid fatalities were

increased significantly (p<0.005) in wheat aphids without BSS and reduced in aphids harbouring BSS (Fig. 2a & 2b). The mortalities among wheat aphids without BSS in choice tests were 30% more than the mortalities in wheat aphids harbouring BSS. This difference in % mortalities of wheat aphids with and without BSS was 18% in no choice trials. This larger difference in mortalities indicate that *A. abdominalis* preferred to feed more on wheat aphids without BSS. Furthermore, ratios of corrected % mortality means in wheat aphids lacked *H. defensa* and harboured *H. defensa* in choice trials were significantly (p<0.005) higher than no choice trials as an indicator of host preference behaviour in choice test (Table 1). While the trend of mortalities caused by *A. rhopalosiphi* in clone 5 and clone 7 in response to the presence or absence of *H. defensa* and *R. insecticola* was similar as we have seen in our no choice trials (Fig. 2a & 2b). The difference in % mortalities of wheat aphids with and without BSS was 19% in choice trials while it was 18% in no choice trials clone 5 sublines. In case of clone 7 sublines, the results of mortalities were same as we have observed in no choice trials. Additionally, ratios of corrected % mortality means in wheat aphids lacked *R. insecticola* and harboured *R. insecticola* in choice and no choice trials were not different significantly (p>0.005). It indicates that *A. rhopalosiphi* did not prefer or rejected the wheat aphids on the basis of presence or absence of BSS (Table 1). All control treatments showed zero mortality against host feeding by *A. abdominalis* while parasitism by *A. rhopalosiphi* caused nadir mortalities in controls (Fig. 2a & 2b).

Host preference behaviour of parasitoids in response to clonal variation

We further studied *A. abdominalis* host feeding and *A. rhopalosiphi* parasitism responses towards the wheat aphid host clonal or genotypic variations. The result showed that genetic or clonal variations among clone 5 and 7 with and without bacterial symbionts are also playing role to resist host feeding of *A. abdominalis*. Wheat aphids of clone 5 and -5 were significantly (p<0.005) less fed by *A. abdominalis* as compared to clone 7 and -7 irrespective of their bacterial facultative symbionts (Fig. 3a & 3b). The mortalities in wheat aphid clones +5 and -5 were 11-13% less as compared to mortalities in wheat aphids of clones +7 and -7. The genotypic expression of clone 5 present more vigorous response against *A. abdominalis* host feeding in presence or absence of bacterial facultative symbionts. When *A. rhopalosiphi* was exposed to the same genotypes of wheat aphids, it did not response towards the genotypic expressions of the clones 5 and 7 in both of the choice trials. Wheat aphid % mortalities did not vary significantly (p>0.005) between these two clonal lines 5 and 7 with

and without BSS (Fig. 3a & 3b). All control treatments showed the zero or negligible dead aphids as compared to parasitoid treatments (Fig. 3a & 3b).

Impact of BSS on larval development of parasitoids

Parasitoids development fitness is greatly affected by presence or absence of bacterial secondary symbionts in the host aphids [41,47]. LSD comparison of % increase in the tibia length of hind leg of the adults, emerged from wheat aphids, in response to absence of *H. defensa* showed that *H. defensa* equally stressed the development of both parasitoids. But the impact of *R. insecticola* was significantly (p<0.005) different for both of the parasitoids (Fig. 4). *R. insecticola* suppressed the development of *A. abdominalis* in clone 7 as compared to the development of *A. rhopalosiphi*.

Horizontal transmission of BSS via parasitoids

Diagnostic PCR, for DNA extracted from parasitoids removed from no choice tests after 24 hours, showed that parasitoids were not infected with any of the bacterial facultative symbionts from wheat aphids harbouring BSS. *A. abdominalis* and *A. rhopalosiphi* emerged from mummies of wheat aphid clones +5, -5, +7, and -7 were also used to extract DNA for diagnostic PCR. The result was not different from the previous PCR result. It was obvious from PCR results that both of the parasitoids are not involved in horizontal transmission of BSS in *S. avenae*.

Discussion

To date, secondary symbiotic bacteria, in aphid populations, are widely known for variety of protective jobs for their hosts [48] but genetically identical host aphid possessing a certain strain of symbiotic bacterium may or may not vary in its resistance to different parasitoid species [41,49] and it can affect host ranges for parasitoid species. Parasitoid host range is influenced by ecological responses of hosts [32] which are mediated by presence or absence of BSS [50]. Particular aphid-symbiont system may decide the host resistance specificity in a particular aphid-parasitoid interaction [42]. *H. defensa*, the most familiar bacterial secondary endosymbiont among aphids, is widely known to confer resistance against many hymenopteran parasitoids of aphids in addition to genetic variations among aphid hosts [4,23,51]. Our no choice trials did also revealed that *H. defensa* imparted protection to wheat

aphids through partial resistance against *A. abdominalis* host feeding and *A. rhopalosiphi* parasitism is a general trend but *R. insecticola* differed in its resistive response conditional to genetic variations of the parasitoids involved in these specific host-parasitoids interactions. The partial resistance imparted to wheat aphids (genetically identical) by of *H. defensa* was consistent against both of the parasitoid species used which is in contradiction to the finding by Łukasik that *H. defensa* does not improve the defensive mechanism against parasitism by *Aphidius ervi* and *Ephedrus plagiator* in *S. avenae* [52] while *H. defensa*, in pea aphids, was highly resistant against *Aphidius eadyi* and weakly resistant against *Aphidius ervi* [10]. This reconfirms that *H. defensa* can or cannot impart resistance to host aphid species against parasitoids genotypes but it depends upon the genetic backgrounds of the partner species involved [23,35,42,49,51,51]. Even in genetically identical *A. fabae*, protection responses of the same *H. defensa* strain vary significantly against clonal lines of *L. fabarum*, a parasitoids of *A. fabae* [23,35,41,42,49,53]. *R. insecticola* was significantly resistant against host feeding by *A. abdominalis* but it did not show any resistance against parasitism done by *A. rhopalosiphi* unlike to *H. defensa*, but genetically identical peach aphids, *Myzus persicae* possessing a particular strain of *R. insecticola* were absolutely resistant against two different parasitoid species *Aphidius colemani* and specificity in our study by providing protection against *A. abdominalis* and *A. rhopalosiphi* parasitoids which was reflected as significant aphid-parasitoid interaction variation. So, the composition of aphid and parasitoid populations might be highly dynamic in local adaptations mediated by the endosymbiont communities in the host aphids. BSS can influence the parasitoid community structure in local biological system conditional to their protection services and long exposures can arouse genetic variations among parasitoid populations to overcome this resistance [41,54].

Parasitoids prefer the best host by choosing aphids with lowest developmental cost over high cost aphids [55]. They are also able to discriminate the aphids harbouring BSS from those who are lacking them and modify their actions accordingly [56] to subsidise their feeding and reproduction. It might be possible that BSS can interfere the host preference behaviour of parasitoids to aid their hosts. So, in our choice trials, *A. abdominalis* significantly preferred wheat aphids lacked BSS over wheat aphids harboured BSS when it has an opportunity to choose between them in clone 5 and 7 respectively but *A. rhopalosiphi* did not exhibit this host preference behaviour. The trend of *A. rhopalosiphi* to exploit the host aphids was same as they have used the wheat aphids in no choice trials. Especially, *R. insecticola* in clone 7 was indifferent even to resist *A. rhopalosiphi* parasitism while it changed the feeding mode of

A. abdominalis significantly. We conclude from these results that *A. abdominalis* prefers wheat aphids devoid of BSS over wheat aphids with BSS, which is more likely an escape from injuries caused by bacterial endosymbionts but *A. rhopalosiphi* response was vice versa. As no previous studies are available, we can relate it to the above discussion that specificity in genotype by genotype interactions are responsible for such behaviour deviations as well and BSS are modifying such preference behaviour deviations. Furthermore, host selection by parasitoids mainly depends upon environmental and host factors determined by chemical and physical interactions [57] which are mediated by bacterial endosymbionts [50].

Natural defences against susceptibility to parasitoids mainly depend upon genetic variations among target host populations [39,58] which in turn change the phenotypic plasticity of the parasitoid populations [59]. Can host genetic variations resist the infectivity of parasitoids? This question has been addressed by many researchers with a conclusion that such genetic differences can also protect the aphids against parasitoids [23,30,39,58,59]. We tried to unveil this interaction in host feeding interaction of *A. abdominalis* and parasitism by *A. rhopalosiphi* by using genetically different clones +5 and +7 and clones -5 and -7 in a different way by choice assays. Interestingly *A. abdominalis* preferred to feed more on clone 7 with and with BSS than on clone 5. Wheat aphid clones +5 and -5 were significantly ($p < 0.05$) resistant to host feeding by *A. abdominalis* as compared to clones +7 and -7. *A. rhopalosiphi* was indifferent towards host selection being genetically different. It is inferred that it can be result of specificity in genotypic interactions because we used genetically different aphid clones with and without different BSS. So, we can assume that the genetic variations can modify the host feeding or parasitism preference behaviour of parasitoids in a specific way.

If parasitoid lays eggs after overcoming host resistance, offspring have to develop in anyway for its survival but offspring suffer with reduced fitness in terms of low emergence, decreased body weight or increased development period [41,47]. This may change the selection or preference behaviour of female parasitoids to avoid host aphids harbouring defensive symbionts [56]. Yet, few evidences about lower development of parasitoid larvae in BSS infected aphids are available [41,47]. But these studies were restricted to two species of parasitoids *Aphidius ervi* and *L. fabarum* in terms of their body mass while developing in pea and bean aphids. Impact of BSS was estimated on development of both parasitoids in wheat aphids in terms of length measurement of tibia from hind legs. Our results suggest that, although parasitoids can overcome host resistance mediated by the presence of BSS but their

development fitness was reduced significantly (p<0.05) as BSS exerted negative effect on them by shorting their bodies. The length of hind tibia was significantly decreased in aphids harbouring BSS as compared to those lacking BSS. But this effect was also specific to genotypes involved in the interactions as *A. abdominalis* suffered equally by both bacterial endosymbionts but *A. rhopalosiphi* was negatively affected by *H. defensa* only.

Maternal or vertical transmission of BSS is thumb rule in aphid populations by manipulating the host reproductive machinery but horizontal transmission is also reported occurring rarely at very low rate [60–62]. This is why sometimes closely related BSS strains may also occur in distant relative of the insect host [63]. The potential routes for horizontal transmission of BSS in aphids can be sexual transfer [64]), transfer by parasitoids [61] and ingestion [65]. It is very rare or nadir for parasitoids to acquire BSS by host feeding on infected host aphids or during development in aphis hosts harbouring BSS [60]. In agreement to this study, *A. abdominalis* and *A. rhopalosiphi* did not show any uptake or contamination of BSS after exposure or emergence from wheat aphids harbouring BSS. It is suggested that these parasitoids did not present a common route for horizontal transmission of BSS among wheat aphids because it would have a negative effect on their own survival and fitness.

Unfortunately, no previous studies are available on host feeding aphid-parasitoid interactions so far. Here, genetically identical wheat aphids were used, hence it is inferred that facultative bacterial endosymbionts are driving the host selection process by parasitoids. Genotypic specificity of bacterial symbionts might be a critical factor to reshape the aphid-parasitoid ecological interactions with a high impact on host ranges of parasitoid species but parasitoids also possess genetic variations which may help them to overcome symbiotic protection aphids with recurrence of exposure [54,66]. It might also be concluded that bacterial symbionts may response differently toward different modes of risks to host aphids like host feeding or parasitism. Their evolutionary role to influence the parasitoids with regard to these findings remains open for future research.

Acknowledgments

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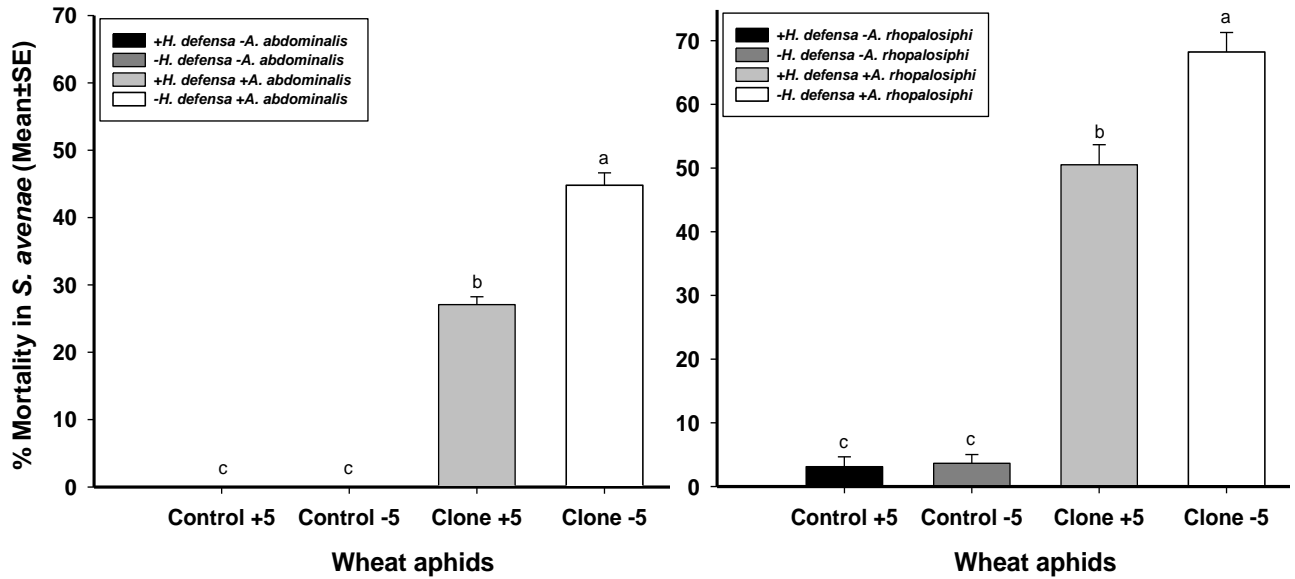
Fig. 1a. % mortality in *S. avenae* (with and without *Hamiltonella defensa*) in response to host feeding and parasitism (no choice trial)
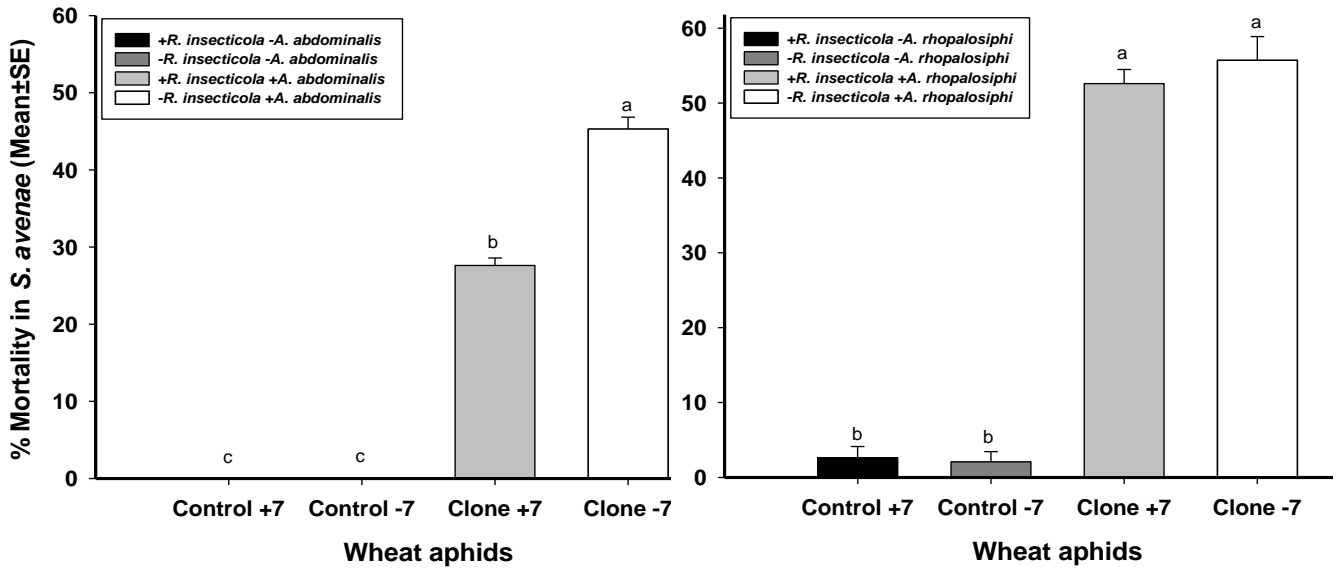

Fig. 1b. % mortality in *S. avenae* (with and without *Regiella insecticola*) in response to host feeding and parasitism (no choice trial)
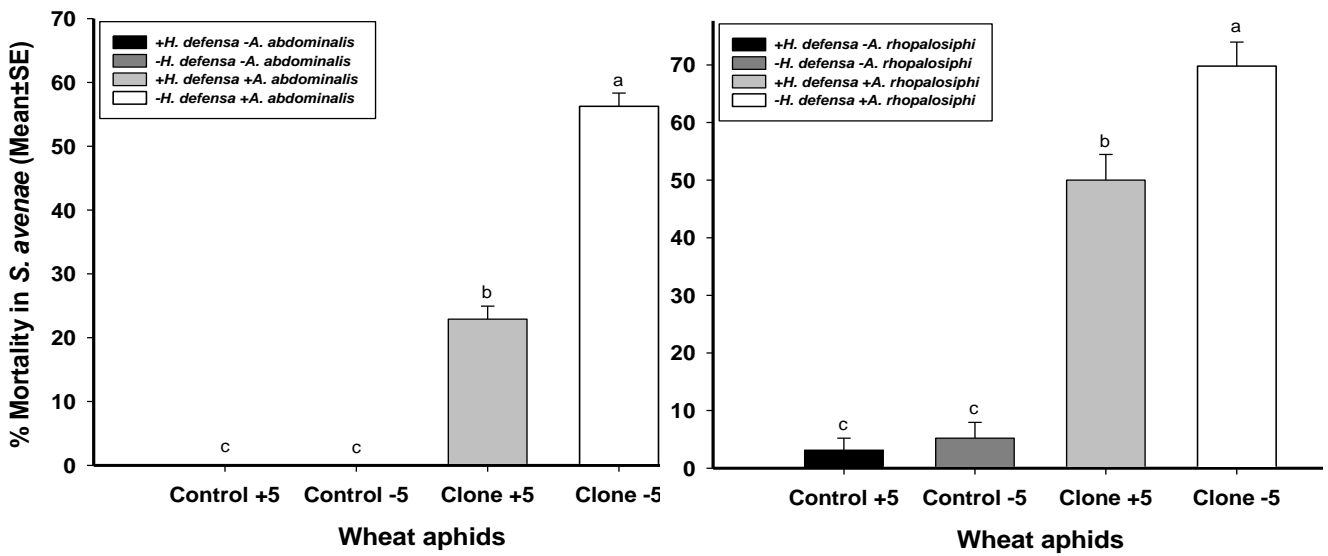
Fig. 2a. % mortality in *S. avenae* (with and without *Hamiltonella defensa*) in response to host feeding and parasitism (choice trial)
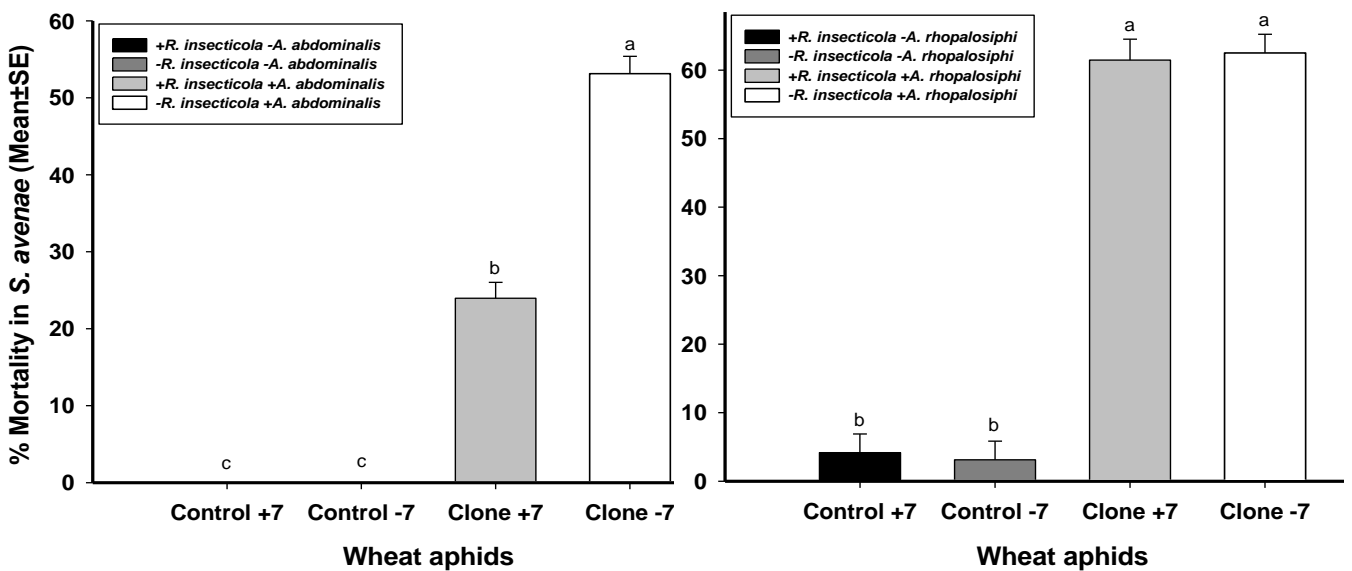

Fig. 2b. % mortality in *S. avenae* (with and without *Regiella insecticola*) in response to host feeding and parasitism (choice trial)
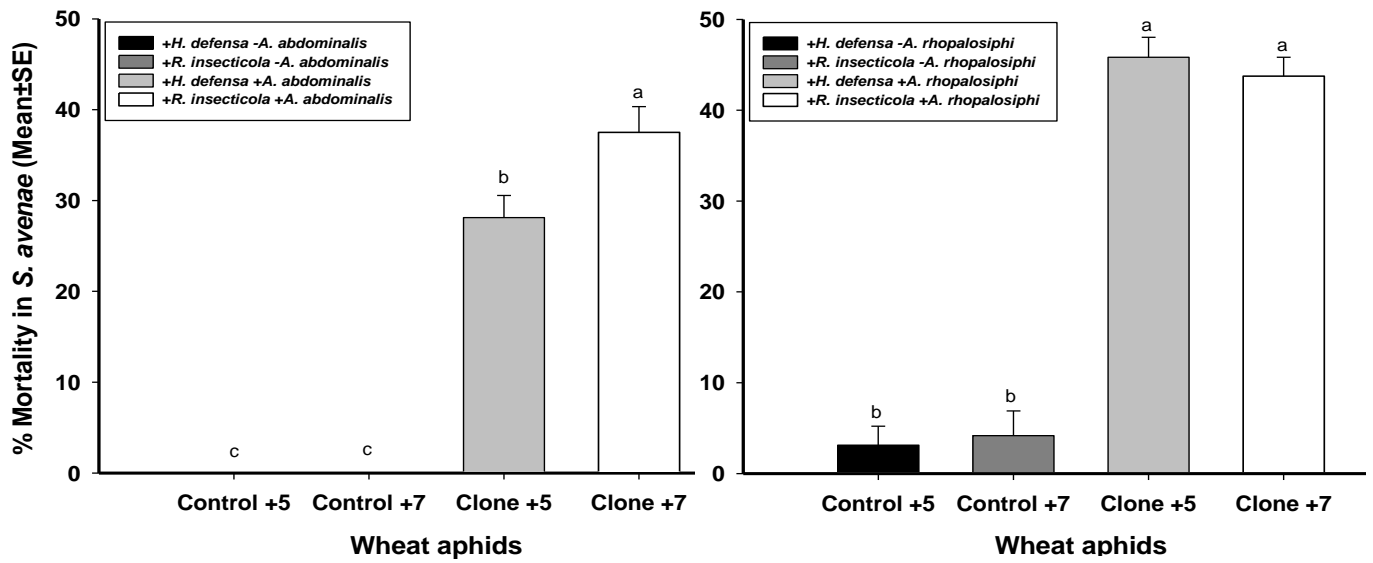
Fig. 3a. % mortality in *S. avenae* (with *Hamiltonella defensa* and *Regiella insecticola*) in response to host feeding and parasitism (choice trial)
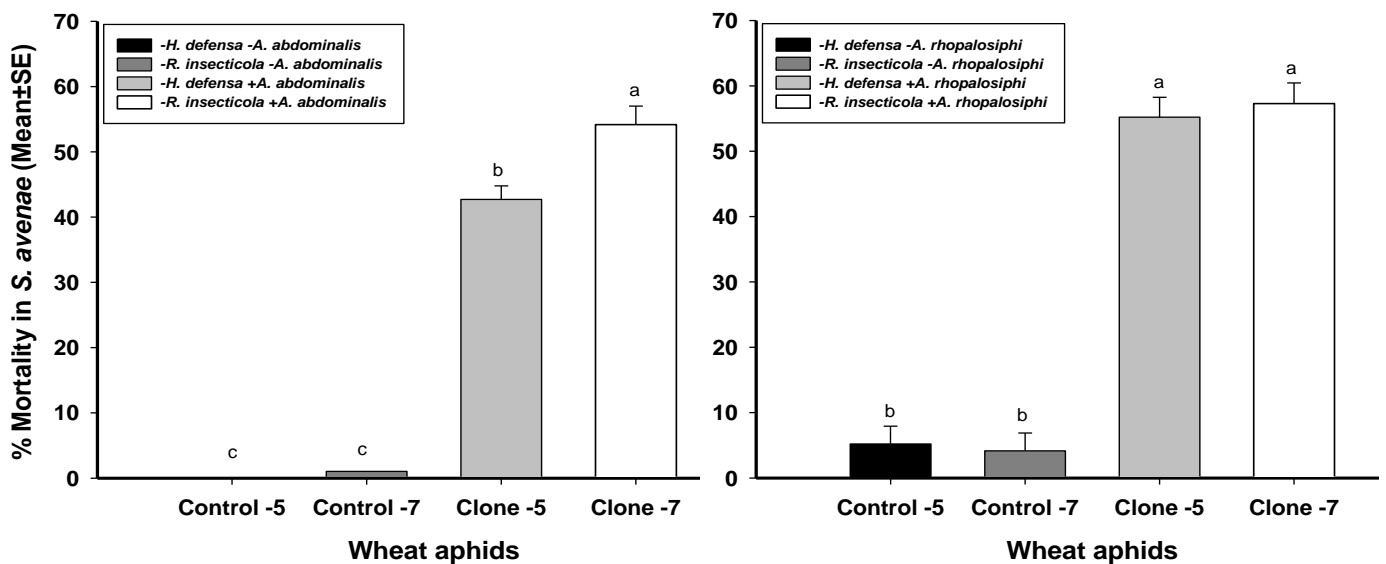

Fig. 3b. % mortality in *S. avenae* (without *Hamiltonella defensa* and *Regiella insecticola*) in response to host feeding and parasitism (choice trial)
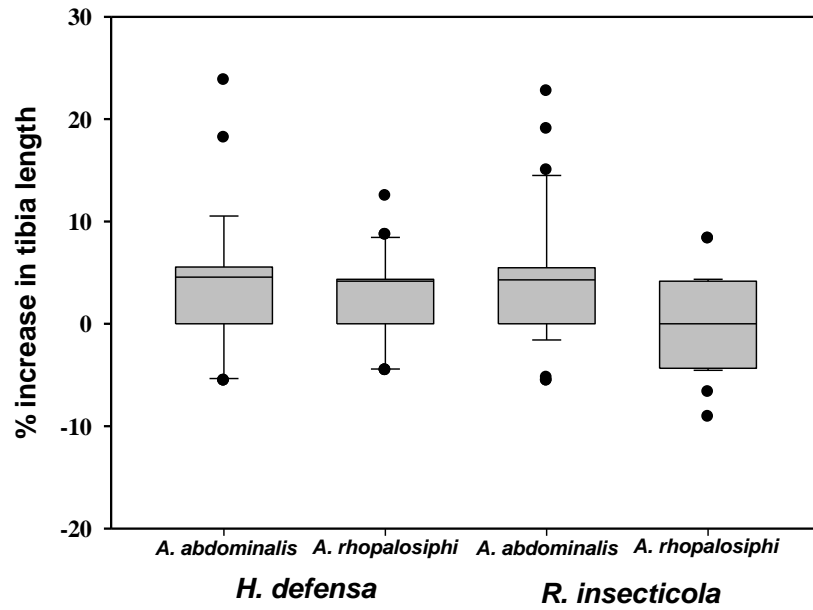
Figure 4: % increase in hind tibia length of parasitoids developed in *S. avenae* (without *Hamiltonella defensa* and *Regiella insecticola*)

Chapter 5

General Discussion and Conclusion

Literature is piled up with studies on effects of bacterial endosymbiont infection on insect hosts. These effects are divided into reproductive manipulation and mutualism. The beneficial effects facilitate the wide spread of bacterial endosymbionts over host insect populations, by increasing symbiont infected host frequencies at the cost of uninfected ones (Oliver & Moran, 2009). Extensive work has been reported to show that bacterial secondary endosymbionts confer resistance to insect hosts against predators, pathogens and parasitoids (Haine, 2008; Brownlie & Johnson, 2009; Oliver & Moran, 2009). More focused work is needed to widen our understanding about the quick spread and broad occurrence of bacterial endosymbiont in insect populations.

The benefits of bacterial secondary endosymbionts may vary in a context depending upon environmental factors. Maintenance of endosymbionts is correlated to the presence of natural enemies because it is costly to have and maintain them. Otherwise, facultative endosymbionts may be vanished from host insect populations (Feldhaar, 2011). Thus, their persistence in insect populations requires diverse fitness benefits with no or minimal cost for their quick and wide dispersal in host insects (Oliver et al., 2008). In case of parasitoid-aphid-endosymbiont interactions, research workers focused on parasitism studies in the last decade. This study was designed to explore the possibilities that facultative endosymbionts may also confer resistance against host feeding behaviour of parasitoids with regard to wheat aphid *Sitobion avenae* (F.), *Aphelinus abdominalis* (Dalman) and *Aphidius rhopalosiphi* interactions.

Hymenoptera parasitic wasps host feeding directly depends upon host population densities (Rosenheim & Rosen, 1992) to secure protein nutrients for better fecundity and longevity (Kidd & Jervis, 1989). We demonstrated for first time in our study that *A. abdominalis* host feeding linearly depends upon the wheat aphid host densities but it is also negatively affected by the presence of defensive bacterial endosymbionts *H. defensa* and *R. insecticola*. The symbiont-host density interactions did not affect the aphid-parasitoid host feeding interactions at all the population levels tested. In addition to defensive bacterial endosymbionts, host genetic variations were also able to influence the host preference behaviour of *A. abdominalis*.

Temperature is one of the most important factors influencing the outcome of aphid-parasitoid interactions. Environmental variables like temperature may influence the decisions of female

parasitic wasps for host feeding (Hansen & Jensen, 2002). Bacterial endosymbionts persistently conferred resistance to wheat aphids against host feeding damages done by *A. abdominalis* at all the temperatures provided but host feeding was positively correlated with increasing temperatures. Host preference behaviour of *A. abdominalis* was not affected by temperature variations.

H. defensa did also confer resistance against parasitism of *A. rhopalosiphi* but *R. insecticola* was not able to serve this function in wheat aphids. Bacterial endosymbionts might be one of the important factors in reshaping the ecological responses and evolutionary processes on the side of parasitoids in aphid-parasitoid interactions. The degree of defensive specificity provided by facultative endosymbionts to host aphids exerts a strong influence on host population for resistance variations and determines the evolution of host ranges for a certain parasitoid. In our study, degree of protection conferred by the two bacterial endosymbionts *H. defensa* and *R. insecticola* largely depended on the genotypic backgrounds of the attacking parasitoids. This could be a possible explanation that why most of the parasitoids specialize on a single or few aphid hosts and it is also a way for the maintenance of genetic variations (Vorburger, 2014). Bacterial endosymbionts may increase the genetic specificity in aphid-parasitoid interactions.

This defensive specificity also explain that a parasitoid genotype should able to recognize and establish after disseminating host variations for bacterial endosymbionts and genotypes in genetically variable populations (Oliver et al., 2012). This was clearly demonstrated when two genetically different parasitoids *A. abdominalis* and *A. rhopalosiphi* were exposed to genetically identical wheat aphids with and without bacterial endosymbionts or genetically different wheat aphids in choice tests. The same genotypic specificity was also depicted through parasitoids developmental variations in wheat aphids infected by same strains of bacterial symbionts. This genotype-genotype specificity has been resulted from particular defensive bacterial endosymbionts existing with same genetic background of their hosts and different genetic background of parasitoids (Schmid et al., 2012).

Based on our findings, we might conclude that *H. defensa* and *R. insecticola* not only confer resistance to their hosts against parasitism but also against host feeding strategy of *A. abdominalis* in wheat aphids. Parasitoid can still be negatively affected by BSS during development after parasitizing wheat aphids. In addition, BSS and genetic variations both can change the host preference behaviour of parasitoids. These fitness advantages confer to their host aphids by these endosymbiotic bacteria expound the adaptive significance of their

heritable and successful spread over many insect populations especially in aphids. It is also inferred that bacterial endosymbionts may drive host selection process by parasitoids depending upon genotype-by-genotype specificity. Defensive role of bacterial endosymbionts is problematic in biological control of aphid pests and the outcome of parasitoid-aphid-endosymbiont interactions may reshape biological control success and should be addressed for risk assessments while formatting IPM policies. More ecological trait studies at population level with regard to prevalence of bacterial endosymbionts will uncover the more subtle effects in future.

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