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Ecological and Physiological Effects of Facultative Endosymbionts of Aphids in the Context of Resistance to Parasitoid Wasps and Immunity

Chen Luo

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THÈSE DE DOCTORAT

Effets écologiques et physiologiques des
endosymbiotes facultatifs dans la résistance aux
guêpes parasitoïdes et l'immunité des pucerons

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Institut Sophia Agrobiotech

Présentée en vue de l'obtention du
grade de docteur en Sciences de la Vie
et de la Santé (Biologie des Interactions
et Écologie) de l'Université Côte d'Azur

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Effets écologiques et physiologiques des endosymbiotes facultatifs dans la résistance aux guêpes parasitoïdes et l'immunité des pucerons

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Résumé

Les symbioses sont omniprésentes dans la nature. Dans ces associations intimes et prolongées entre différents organismes, l'effet de l'expression des gènes d'un partenaire sur l'autre peut conduire à l'apparition de nouveaux phénotypes, un concept appelé "phénotype étendu". Ma thèse porte sur l'étude des interactions hôte-parasitoïde-symbiotes chez les pucerons, principalement le puceron du pois *Acyrtosiphon pisum*, devenu un modèle de par sa symbiose nutritionnelle obligatoire avec *Buchnera aphidicola* et facultative avec un ou plusieurs symbiotes, les plus courants étant *Hamiltonella defensa* (*Hd*), *Regiella insecticola* (*Ri*) et *Serratia symbiotica* (*Ss*). Le travail présenté aborde des aspects écologiques et physiologiques de la symbiose facultative chez les pucerons. Les pucerons sont des hôtes pour une communauté complexe de parasitoïdes qui s'inscrivent dans l'hypothèse Performance-Preference (PPH) selon laquelle les femelles vont préférentiellement pondre dans les hôtes qui maximisent la survie et les performances de leur progéniture. L'évaluation de la PPH permet de classer les parasitoïdes en termes de degré de spécialisation. J'ai participé à la détermination de la PPH de trois parasitoïdes (*Aphelinus abdominalis*, *Aphidius ervi* et *Diaeretiella rapae*) en utilisant 12 espèces de pucerons (6 Aphidini et 6 Macrosiphini) maintenus sur différentes plantes hôtes et dont le statut symbiotique était établi. *A. abdominalis* et *D. rapae* sont apparus comme des généralistes et *A. ervi* comme un spécialiste modéré. Toutes les espèces ont montré une faible sélectivité vis-à-vis de l'hôte quelle que soit la plante hôte ou le symbiote, mais le succès parasitaire était impacté par certains symbiotes. J'ai ensuite étudié l'effet du génotype de l'hôte, des génotypes (hôte x symbiote) sur la réussite des parasitoïdes sur des clones du puceron *Sitobion avenae* infectés artificiellement avec une souche protectrice de *Ri*. Les lignées infectées sont de meilleurs hôtes pour *Aphelinus asychis* mais pas *Aphidius gifuensis*, par rapport aux mêmes clones exempts d'infection. L'effet de *Ri* est donc dépendant de l'espèce parasitoïde, indiquant que le coût/bénéfice d'un symbiote dépend du contexte. Dans la seconde partie de thèse, je me suis concentré sur le système immunitaire de l'hôte en tant que facteur central dans l'établissement et l'évolution des interactions entre les organismes. L'annotation de différents génomes de pucerons montre une immunité réduite qui pourrait être due à leur adaptation à une vie symbiotique. Les hémocytes et l'activité phénoloxydase, deux composants immunitaires majeurs, ont été décrits chez le puceron. J'ai développé des outils moléculaires pour analyser l'expression de gènes codant pour les deux phénoloxidasés (PO) d'*A. pisum* (PO2 et PO2-X1) et pour estimer leur quantité dans l'hémolymphe. J'ai utilisé des clones de différents fonds génétiques sans symbiote secondaire (LL01, YR2-Amp, T3-8V1-Amp) et les lignées naturelles ou artificielles YR2 ou T3-8V1 infectées par *Hd*, *Ri* ou *Ss*. J'ai démontré que : i) les deux gènes sont exprimés et que leurs produits sont présents sous une forme circulante dans l'hémolymphe, ii) l'expression des gènes, la quantité et l'activité de la PO sont fortement corrélées et dépendent du fond génétique de l'hôte et iii) ces trois marqueurs sont significativement diminués par la présence de *Hd* et de *Ri*. J'ai observé une corrélation entre l'impact des facteurs de stress sur les traits de vie du puceron et la présence de certains symbiotes (et donc la quantité de PO des pucerons), mais pas de corrélation avec la variation de PO après le stress. Ce travail montre donc une forte interaction entre la capacité immunitaire de l'hôte et le statut symbiotique du puceron, et il peut expliquer le succès ou l'échec de certains parasitoïdes qui ne sont pas hautement spécialisés pour l'hôte qu'ils attaquent.

Mots clefs

Pucerons, Symbiose, Ecologie, Interactions, Parasitoïdes, Hypothèse preference performance, Immunité, Phénoloxydase, traits d'histoire de vie

Summary

Symbiosis is omnipresent in nature. In these intimate and prolonged associations between different organisms, the effect of gene expression from one partner on the other can lead to the appearance of new phenotypes, a concept called "extended phenotype". My thesis focuses on the study of host-parasitoid-symbiont interactions in aphids, mainly the pea aphid *Acyrtosiphon pisum*, which has become a model for its obligatory nutritional symbiosis with *Buchnera aphidicola* and facultative with one or more symbiotes, the most common being *Hamiltonella defensa* (*Hd*), *Regiella insecticola* (*Ri*) and *Serratia symbiotica* (*Ss*). My work addresses ecological and physiological aspects of facultative symbiosis in aphids. Aphids are hosts for a complex community of parasitoids that fit in the Performance-Preference Hypothesis (PPH) suggesting that females will preferentially lay eggs in hosts that maximize the survival and performance of their offspring. The PPH evaluation classifies parasitoids in terms of degree of specialization. I participated in the determination of the PPH of three parasitoids (*Aphelinus abdominalis*, *Aphidius ervi* and *Diaeretiella rapae*) using 12 aphid species (6 Aphidini and 6 Macrosiphini) maintained on different host plants and whose symbiotic status was established. *A. abdominalis* and *D. rapae* appeared as generalists and *A. ervi* as a moderate specialist. All species showed low selectivity towards the host regardless of the host plant or symbiont, but parasitic success was impacted by some symbionts. I then studied the effect of host genotype, genotypes (host x symbiont) on parasitoid success using artificially infected clones of the aphid *Sitobion avenae* with a protective strain of *Ri*. Infected lines are better hosts for *Aphelinus asychis* but not *Aphidius gifuensis*, compared to the same infection-free clones. The *Ri* effect is therefore dependent on the parasitoid species, indicating that the cost/benefit of a symbiont is context-dependent. In the second part of my thesis, I focused on the host's immune system as a central aspect in the establishment and evolution of interactions between organisms. The annotation of different aphid genomes shows a reduced immunity that could be due to their adaptation to a symbiotic life. Hemocytes and phenoloxidase activity, two major immune components, have been described in aphids. I have developed molecular tools to analyze the expression of genes encoding both phenoloxidases (PO) of *A. pisum* (PO2 and PO2-X1) and to estimate their amount in the hemolymph. I used clones from different genetic backgrounds without secondary symbiont (LL01, YR2-Amp, T3-8V1-Amp) and natural or artificial lines YR2 or T3-8V1 infected with *Hd*, *Ri* or *Ss*. I have demonstrated that: i) both genes are expressed and their products are present in circulating form in the hemolymph, ii) gene expression, amount and activity of PO are highly correlated and depend on the genetic background of the host and iii) these three markers are significantly decreased by the presence of *Hd* and *Ri*. I observed a correlation between the impact of stressors on the aphid's life traits and the presence of some symbionts (and therefore the amount of PO in aphids), but no correlation with the variation in PO after stress. This work therefore shows a strong interaction between the host's immune capacity and the symbiotic status of the aphid, and can explain the success or failure of some parasitoids that are not highly specialized for the host they attack.

Key words

Aphids, Symbiosis, Ecology, Interactions, Parasitoids, Preference performance hypothesis, Immunity, Phenoloxidase, Life-history traits

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INTRODUCTION

A fundamental attribute of life on earth is symbiosis

Symbiosis, from Greek meaning “living together”, was used in the late 19th century to designate two species that live a long-term intimate association. It was first used by Albert-Bernhardt Frank and Anton de Bary to describe the relationship between algae and fungi in lichens (Frank 1877; de Bary 1879; For an historical review see Perru 2006). Frank and de Bary used “symbiosis” to refer to all types of species interactions that can be divided into three categories based on whether the symbiont has beneficial (e.g. mutualism), neutral (e.g. commensalism) or detrimental (e.g. parasitism) effects for the host (Brownlie and Johnson 2009) (see Table 1 for definition of terms).

Although it was in 1927 that Ivan E. Wallin hypothesized the bacterial origin of mitochondria and the formation of new species primarily through the acquisition of bacterial endosymbionts¹ (Wallin 1927), it was not until the late 1960s that Lynn Margulis brought this topic back to attention when she proposed that three fundamental organelles, the mitochondria, the chloroplast and the (9+2 pattern) basal bodies of flagella, were themselves formerly free-living (prokaryotic) cells (Sagan 1967).

She further defined the concept of holobiont (Margulis 1991), animal or host plant, as well as all associated microorganisms living on or in it (exo- and endosymbionts) (Rosenberg and Zilber-Rosenberg 2018; Simon et al. 2019). The holobiont therefore challenges the notion of individuality, an individual being defined not only by his own genome, but also by his symbionts including its microbiome (all the microbes associated with an animal or a plant). The concept of hologenome (used to describe the collection of genomes of a host and its microbiota) considers that all holobionts exist on a spectrum ranging from extreme symbiosis (obligatory) to a state of looser symbiosis.

The endosymbionts within their host are usually an example of extreme mutual metabolic and genetic adaptation with clear vertical transmission. Humans with their exosymbionts, however, are an example of symbiosis and mode of transmission much more complex and seemingly looser.

¹ He quotes in the introduction of his book “the author has investigated the nature of mitochondria and has arrived at the unqualified conviction that these bodies in the cell are bacterial in nature” and further “A study of the literature on "micro-symbiosis" revealed a wealth of evidence that supports and emphasizes the significance of bacteria in the origin of species”.

Table 1. Quick reference of terms and definitions. Adapted from Tipton et al. (2019)

Term	Definition
Symbiosis	Interaction among species
Host	Defined as the provider of resources
Symbiont	Consumer of host resources that may or may not provide services in return
Endosymbiont	Any organism that lives within the body or cells of another organism
Exosymbiont	A symbiont that lives outside the body of its host
Holobiont	Assemblage of participants in a symbiosis
Commensalism	Type of symbiosis where one partner benefits without any measurable effect on the other
Mutualism	Type of symbiosis where both partners benefit
Parasitism	Type of symbiosis where one partner benefits at the expense of the other
Resistance	A stable state property, a community's tendency to remain in its current state
Syntrophy	Type of mutualistic symbiosis where all partners depend on each other metabolically

Although the literature somehow disagrees on the use of the words symbiosis and symbiont, symbiosis is widely used to describe the intricate relationship between two or more species living in intimate (involving physiological) interaction and it is recognized as a central driver of evolution through the whole tree of life (Brucker and Bordenstein 2012; Douglas 2014; Hurst 2017; Rosenberg and Zilber-Rosenberg 2018). It is now accepted that symbiosis is more of a continuum between mutualism-commensalism-parasitism since the cost of the interaction for the host may depend on the conditions. In ecology, the term "symbiosis" encompasses a large range of species interactions (such as predation, competition, etc.) that can be classified according to their outcome in terms of fitness cost to the host. This relationship could therefore vary from a relatively "simple" association, consisting of a host and one or more partners, to a remarkably complex one. Indeed, all macro-organisms observed to date are associated with micro-organisms and the symbiosis has entered the era of the meta-organism, an individual (host) with roughly the same number of bacteria and host cells. For example, the human body contains $3-4 \cdot 10^{13}$ bacteria and about $4 \cdot 10^{13}$ cells (Dethlefsen et al. 2007; Sender et al. 2016). These bacteria belong to hundreds or thousands of microbial species whereas only 56 cells categories have been described (Bianconi et al. 2013). In such ecosystems, microbial members engage in a multitude of interactions with each other, including forms of competition, cooperation and exchange of DNA, as well as interactions with the host. This hosted bacterial community could independently or synergistically affect many aspects of host physiology and influence its phenotypes (Esser et al. 2019; Lynch 2019). In addition, the imbalance of the microbiome or dysbiosis is now strongly involved in the genesis of neurologic, metabolic and cellular diseases (Fig. 1) (Rosenberg and Zilber-Rosenberg 2012; Levy et al. 2017; Belizário et al. 2018; Esser et al. 2019). Thus, the interaction between the host and the symbiont challenges the traditional portrayal of species evolution and the manipulation of symbioses can open fascinating new perspectives in health, biotechnology and agriculture.

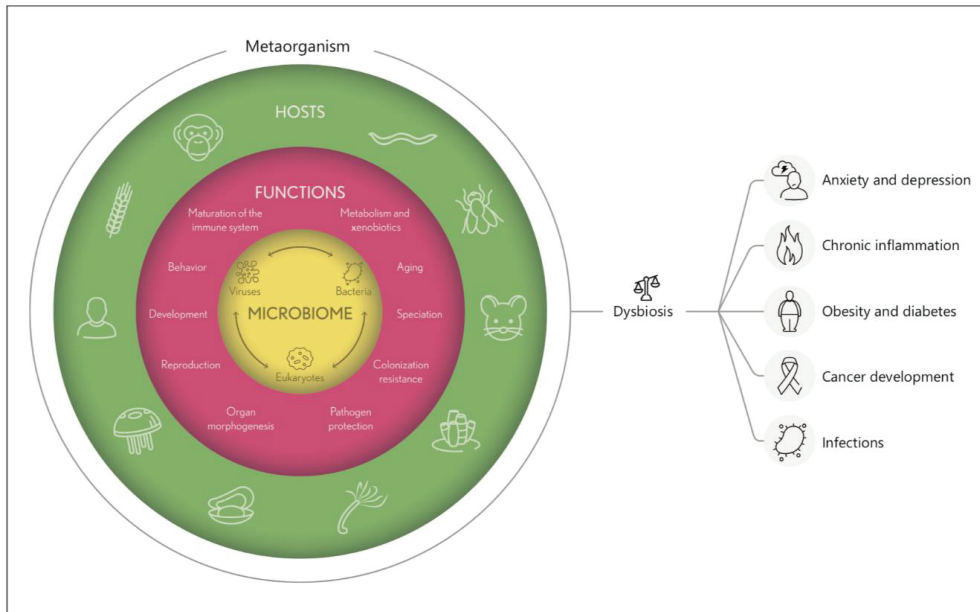


Fig. 1. Functional interactions in meta-organisms. Interdependent relationship of eukaryotic organisms with their microbiome, including bacteria, viruses and other small eukaryotes, and viewed as meta-organisms. Microorganisms can contribute to different host functions such as metabolism, aging, behavior, protection from pathogens, and maturation of the immune system. Dysbiosis or imbalances in these homeostatic host-microbiome interactions can be associated with various diseases in human (from Esser et al. 2019).

Insects symbiosis

Insects are the most abundant and diverse animals on earth, accounting for >90% of known animal species and dominating a variety of terrestrial habitats. Despite the fact that symbionts sequester resources from their hosts and are therefore costly to maintain, there is an extraordinary diversity of them in insects. Some spread through host populations by providing their hosts fitness benefits or manipulating the host sex ratio, but others do not, whose maintenance in host lineages therefore remains an enigma (Richards and Brooks 1958; Douglas 2015). In fact, mutualistic bacteria are probably a key factor in adapting insects to novel environments and food sources, and several insect taxa are entirely dependent for successful growth and reproduction on their mutualistic bacteria that have been passed from mother to offspring for up to several hundred million years (Moran et al. 1993). Most of these symbioses are found in insects that feed on a poor or unbalanced diet such as wood, plant sap or blood. For examples, xylophagous insects such as termites and cockroaches assimilate wood through the metabolic capacities of microorganisms present in their guts (Berlanga 2015); many hemipterans (aphids, whiteflies etc.) are plant sap feeders whose survival and/or reproduction requires nutrient exchange with their hosted intracellular bacteria. Tsetse flies, vectors of African trypanosome parasites, feed exclusively on vertebrate blood. They harbor the obligate

endosymbiont *Wigglesworthia glossinidia* that contributes to its reproduction, nutrition and immunity through the production of vitamins that supplement the meal of vertebrate host blood (Pais et al. 2008; Bing et al. 2017). While the gut is the primary site for symbiotic bacteria, most early studies on insect symbiosis focused on obligate endosymbiosis and pay particular attention to a specialized organ-like structure, the bacteriome (called at the time mycetome), whose sole function seemed to be to house and maintain the symbionts (Buchner 1965; Moran and Telang 1998; Sapp 2002; Baumann et al. 2013; Fig. 2). An estimated proportion of 10%–20% of insect species have a bacteriome, composed of bacteriocytes cells, and among the groups with bacteriocytes-associated obligate symbionts (or “primary” bacteriocytes) are the aphids, tsetse flies, whiteflies, psyllids, mealybugs, weevils, and carpenter ants (Moran and Telang 1998). It is of note that not all obligate symbionts are nutritional: some symbionts have become indispensable for the reproduction of their host and the defense against parasites. For example, *Wolbachia* (*Wolbachia pipientis*) is a maternally-transmitted endosymbiotic bacterium that lives in the cells of arthropods and filarial nematodes (Lo et al. 2007; Zug and Hammerstein 2014). About 40% of terrestrial arthropods (Zug and Hammerstein 2012) are infected by these gram-negative bacteria that greatly influence their sex ratio. These reproductive manipulations (or reproductive phenotypes) include cytoplasmic incompatibility, killing or feminization of genetic males and induction of thelytokous parthenogenesis (Werren et al. 2008). Since *Wolbachia* is exclusively transmitted by the female germline, all reproductive manipulations directly or indirectly increase the proportion of infected females. However, there are examples of *Wolbachia* invading insect populations without impairing reproduction: *Drosophila* flies infected with some of these *Wolbachia* strains are less susceptible to mortality induced by a range of RNA viruses, showing a benefit in terms of fitness for a *Wolbachia*-infected host and therefore explaining its spread (Hedges et al. 2008; Osborne et al. 2009).

There are thus increasing evidence that symbionts can shape insect interactions with the various organisms they encounter from plants to predators and pathogens (Piel et al. 2004; Hedges et al. 2008; Oliver et al. 2003; Łukasik et al. 2013a). By influencing such interactions, symbionts can impact the amount and quality of resources (e.g. food) available to higher trophic levels, which can lead to cascading effects or even collapse of the community (Omacini et al 2001; Sanders et al. 2016). In turn, these phenotypes conferred by symbionts can facilitate the shift of hosts toward new ecological niches, driving diversification (Margulis and Fester 1991; Moran 2002).

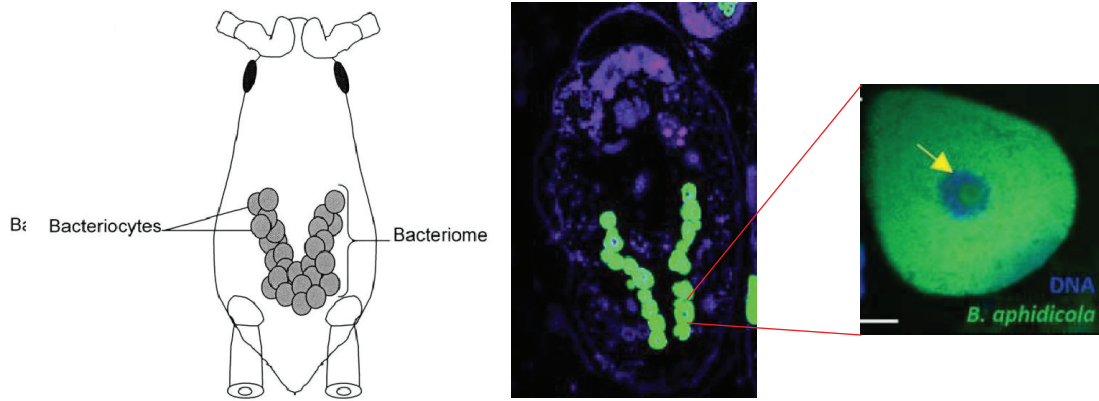


Fig. 2. The bacteriome and bacteriocytes. Left: drawing of a pea aphid adult indicating the position of the bacteriocytes forming the bacteriome that lines the abdomen and surrounds the aphid gut. Center: *Buchnera* (stained in green) highlights each bacteriocyte of the bacteriome. Right: enlargement showing one bacteriocyte filled with *Buchnera* cells (green dot), and the nucleus stained in blue at the center (yellow arrow).

The aphids

Aphids have been studied for a long time because of their peculiar biology and the fact that many species are considered agricultural pests that directly impact plant growth by ingesting phloem and indirectly as vectors of numerous diseases (Kennedy 1950; Day 1955; Sylvester 1980; Ng and Perry 2004). Aphids are small (1-10 mm), soft-bodied insects that feed on plants by inserting their sucking mouthparts called stylets through the epidermis and mesophyll to reach the phloem. Aphids are a monophyletic group with about 4500 species in the order Hemiptera (i.e. the superfamily Aphidoidea). While some aphid ancestors date back 250 million years (My), the majority of Aphidoidea originates from Jurassic about 150 My, parallel to the diversification of angiosperms (Dohlen and Moran 2000). Although phylogenetic relationships within Aphididae are not fully resolved (Ortiz-Rivas and Martínez-Torres 2010), three main families are generally recognized: Aphididae (including so-called true aphids), with viviparous parthenogenetic females, and Adelgidae and Phylloxeridae, with oviparous parthenogenetic females (Fig. 3). During parthenogenetic reproduction, the embryos complete their development within the mother, which gives live birth to first instar nymphs (viviparous parthenogenesis) that develop through four instars nymphs (larval) before molting into adults. As hemimetabolous insects, pea aphids undergo incomplete metamorphosis. Several embryos develop sequentially in each ovariole of the mother and daughters genetically identical to their mother are produced from a single female. Aphids colonies from a single mother are therefore called clones. In addition, late-stage embryos are already developing within female embryos in the mother, so-called generation telescoping.

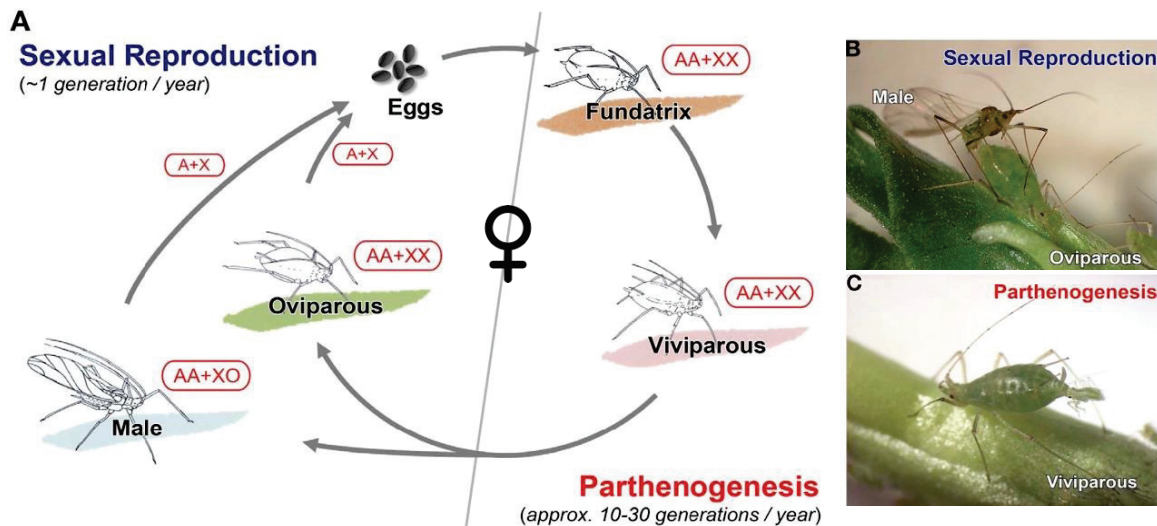


Fig. 3. Typical annual life-cycle of *A. pisum* aphids. (A) Schematic diagram of a typical life cycle of aphids: Aphids reproduce by thelytokous parthenogenesis in spring and summer. In holocyclic life-cycle aphids, males and oviparous (sexual) females appear in late fall and produce fertilized eggs for overwintering. On hatching, each egg develops into a wingless female called "foundress" (= fundatrix) which gives birth parthenogenetically to further parthenogenetic females (= viviparous, virginopares). These can be winged (= alate) or wingless (= apterous), in response to environmental conditions. Overpopulation particularly stimulates the development of the winged form. The number of parthenogenetic generations depends on temperature and other factors; it takes about 7- 10 days from birth to first reproduction at moderate temperatures. Each parthenogenetic female already contains her own granddaughters in an embryonic state. Parthenogenesis in aphids is apomictic, i.e. without meiosis and therefore without genetic recombination. Thus, the offspring of a single foundress are genotypically identical and constitute a "clone", although a number of alternative phenotypes may be expressed. (B) sexual individuals mating (male and oviparous female) and (C) viviparous female giving birth. From Ogawa K. (Ogawa and Miura 2014).

Aphids exhibit impressive flexibility in behavioral and physiological responses to stress. The varied aphid repertoire for coping with stress includes changes in feeding behavior, dispersal, selective resorption of embryos and associations with beneficial symbiotic microbes.

Aphid obligatory symbionts

Aphids feed on sap, mostly phloem sieve elements, a part of the plant responsible for transporting nutrients produced in the leaves by photosynthesis throughout the plant. This poses two main problems: first, the phloem contains high concentrations of simple sugars that aphid guts must convert into long-chain oligosaccharides to avoid osmotic problems. Aphids therefore excrete the excess sugar-rich honeydew. Second, the phloem contains a low and unbalanced spectrum of essential amino acids. Aphids must therefore acquire supplements in these essential amino acids. To do this, most aphids harbor the intracellular bacterium *Buchnera aphidicola*, an alpha-proteobacterium close to *Escherichia coli*. *B. aphidicola* is evolutionarily derived from free-living bacteria and both the aphid and the symbiont must have therefore evolved mechanisms to host the bacterium into specialized cells (bacteriocytes) (Fig. 2) (Buchner 1965; Douglas 1989; Moran et al. 1993; Fukatsu 1994; Baumann et al. 1997; Wernegreen 2002; Moran et al. 2008).

Buchnera endosymbiosis was established about 200 My ago, as evidenced by phylogenies (Moran et al. 1993; Peccoud et al. 2009; Nováková et al. 2013). The symbiont is transmitted vertically from mother to offspring and this combination of clonality and maternal transmission leads to high rates of fixation of mutations, resulting in high rates of genome-wide protein evolution (Moran 1996) as well as gene inactivation (van Ham et al. 2003). One consequence is a significant shrinkage of the genome with the loss of essential genes and a number of protein-encoding genes that is now 354 to 587 (Chong et al. 2019). Despite this reduced genome, *Buchnera* retains genes involved in the biosynthesis of the ten amino acids essential to animals [except for the Lachninae subfamily, of which *Buchnera* has a smaller genome due to the presence of an additional obligate symbiont (Chong et al. 2019)]. *Buchnera* is also associated with two plasmids dedicated to the synthesis of amino acids. The first has a leucine operon (pLeu) (Silva et al. 1998), while the second is for tryptophan (pTrp) (Panina et al. 2001). These two amino acids are essential for the host and these two plasmids therefore play a key role in the symbiotic relationship.

Thus, *Buchnera* synthesizes, in coordination with the host metabolism, the amino acids essential vital for the survival and reproduction of the host (Douglas 1998; Wilson et al. 2010; Colella et al. 2018; Feng et al. 2019) and meets the nutritional needs of aphids by supplementing their low amino acid sap diet.

Aphid facultative symbionts

Aphid are host to several secondary symbionts, but their association varies from free association to co-obligation with intermediate stages of dependence (Russell and Moran 2005; Oliver et al. 2010; Henry et al. 2013; Guyomar et al. 2018). These symbionts have been found in secondary bacteriocytes, free in the hemolymph and more recently in the hemocytes (Baumann et al. 1995; Tsuchida et al. 2005; Oliver et al. 2010; Schmitz et al. 2012) (Fig. 4). As *B. aphidicola*, they are mainly transmitted from mother to offspring (Russell and Moran 2005). Yet, phylogenetic analyses have highlighted frequent horizontal transfers of secondary symbionts between host lineages and acquisition of these symbionts by plants or natural enemies is also possible (Sandström et al. 2001; Henry et al. 2013).

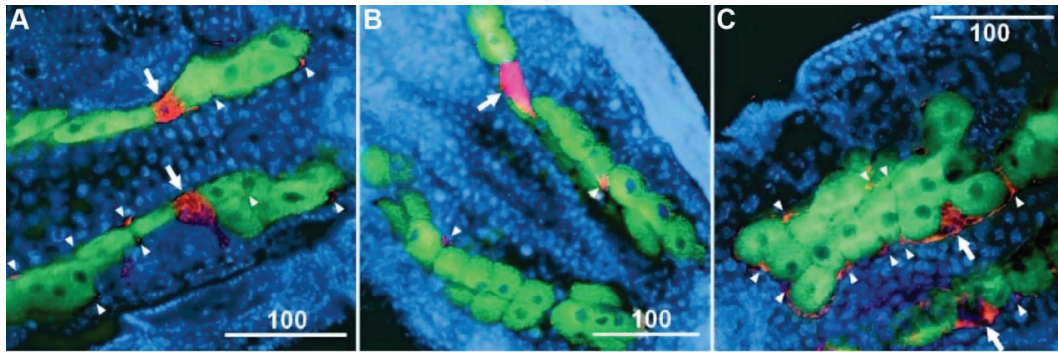


Fig. 4. Distribution of secondary symbionts of *A. pisum* in the tissues of its host. (A-C) Detection by in situ hybridization (FISH) of *B. aphidicola* (green) and *S. symbiotica* (A), *H. defensa* (B) and *R. insecticola* (C) (red) in aphid embryos. Blue signal: cell nucleus; Arrows: secondary symbiont in bacteriocytes; arrowheads: secondary symbiont in "Sheath cells"; scale: 100 μ m. Sheath cells are located at the periphery of the bacteriome and are closely associated with the primary bacteriocytes. Based on (Moran et al. 2005; Dion 2011a).

At least nine symbionts have been described from individual aphids caught in the fields (Fig. 5), the most common being *Hamiltonella defensa*, *Regiella insecticola*, and *Serratia symbiotica*, while the others (*Rickettsiella viridis*, *Candidatus Fukatsuia symbiotica* (previously Pea Aphids X-type Symbiont, PAXS), *Rickettsia*, *Spiroplasma*, *Wolbachia* and *Arsenophonus*) are rare in most populations (Moran et al. 2005; Oliver et al. 2014; McLean et al., 2016; Cariou et al. 2018; Guyomar et al. 2018) or are present only in certain species of aphids (Augustinos et al. 2011). However, the different symbionts of the pea aphid show significant and heterogeneous genotypic diversity, suggesting the existence of a large number of "strains" of symbionts, and different strains of the same symbiont can coexist within the same aphid host (Guyomar et al. 2018).

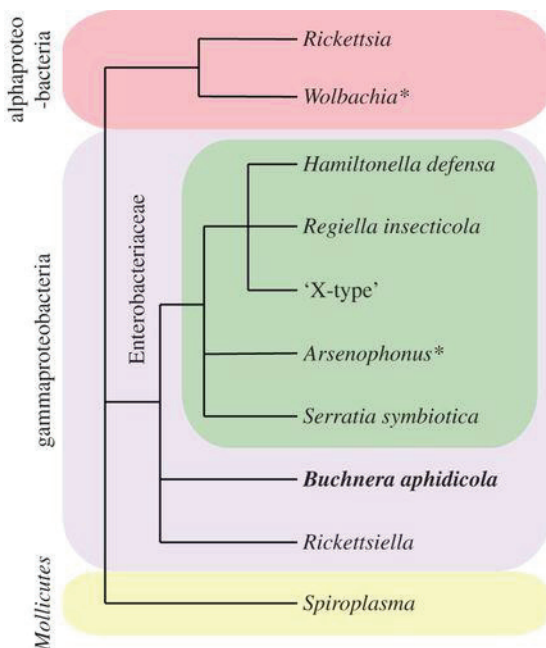


Fig. 5. Taxonomic relationships of aphid bacterial symbionts. The primary symbiont, present in virtually all aphids, is in bold type. The asterisks refer to species of symbionts not found in pea aphids. (From McLean et al. 2016)

In general, these symbionts are not necessary for the aphid's growth and reproduction and are therefore considered "facultative", but they can strongly affect the fitness of aphid hosts through effects on the specialization on the host plant, manipulation of the behavior, resistance to heat, pathogens and parasitoids (Table 2) (Montllor et al. 2002; Oliver et al. 2003, 2010; Tsuchida et al. 2004, 2010; Scarborough et al. 2005; Dion et al. 2011b). However, maintaining a secondary symbiont can also result in fitness costs to the host such as decreased survival and fecundity and modulation of its immunity (Simon et al. 2011; Laughton et al. 2014; Desneux et al. 2018). All these positive or negative effects are ecologically and evolutionarily significant, but they are still poorly understood at the molecular level with the exception of parasitoid resistance. In addition, the phenotypic effect of these bacteria on their host may vary for the same species of symbionts depending on the environmental context. (Leclair et al. 2016a).

Table 2. Main phenotypic effects of pea aphid facultative symbionts.

Symbionts	Phenotypic effects
<i>Hamiltonella defensa</i>	Protection against parasitoids [Oliver et al. 2003] Alteration of defensive behavior [Dion et al. 2011b]
<i>Regiella insecticola</i>	Protection against fungal pathogens [Łukasik et al. 2013a] Adaptation to host plant [Tsuchida et al. 2004]
<i>Fukatsuia symbiotica</i> (PAXS)	Protection against parasitoids [Guay et al. 2009] Heat resistance [Guay et al. 2009]
<i>Serratia symbiotica</i>	Heat resistance [Russell and Moran 2005] Protection against parasitoids [Oliver et al. 2003]
<i>Rickettsia</i> sp.	Protection against fungal pathogens [Łukasik et al. 2013a] Heat resistance [Montllor et al. 2002]
<i>Rickettsiella viridis</i>	Color change [Tsuchida et al. 2010] Protection against fungal pathogens [Łukasik et al. 2013a] Protection against parasitoids [Leclair et al. 2017]
<i>Spiroplasma</i> sp.	Protection against fungal pathogens [Łukasik et al. 2013a] Reproductive manipulation [Simon et al. 2011]

Replacement/complementation of *Buchnera* by secondary symbionts

Aphids lacking *Buchnera* usually suffer growth retardation, sterility, and even quick death (Douglas 1989) and few facultative symbionts examined can compensate for the role of the primary symbiont. In pea aphids, *S. symbiotica* could enable survival and reproduction after complete elimination of *Buchnera*, suggesting that it would supply essential nutrients to the host (Koga et al. 2003, 2007). *Cinara* aphids (Aphididae) typically harbor two obligate bacterial symbionts: *Buchnera* and *Serratia symbiotica*. The genome of *Buchnera* from *Cinara* is much smaller than that of *Buchnera* from the pea aphid and it has lost the capacity to synthesize two essential vitamins, biotin and riboflavin (Lamelas et al. 2011). In terms of these nutrients, these aphids rely on *S. symbiotica* (Lamelas et al. 2011; Manzano-Marin et al. 2016, 2017) but this

co-symbiont has been replaced several times in *Cinara* aphids by other bacterial taxa, including other known facultative symbionts of aphids (e.g. *Fukatsuaia*, *Sodalis*, and *Hamiltonella*) (Meseguer et al. 2017). It may possibly due to that *S. symbiotica* shows a higher frequency of infection in aphids than other facultative symbionts: of the 156 aphid species examined, 74 harbor *S. symbiotica* and, compared to other facultative symbionts, *S. symbiotica* was consistently the highest in proportion throughout the world, with the exception of Australasia (a single aphid species was tested) (Zytynska and Weisser 2016). *S. symbiotica* also has the highest infection rate in a natural aphid population with almost 100% of infected individuals in *Sitobion avenae* (Luo et al. 2016) and 78% in *Macrosiphum rosae* (Desneux et al. 2018). In addition, the infection rate of *S. symbiotica* was always significantly higher than that of the other two common symbionts (*H. defensa* and *R. insecticola*) in the pea aphid, regardless of its native or exotic origin (Desneux et al. 2018).

In addition to *S. symbiotica*, a recent study has shown that *Wolbachia* also plays a key role in the banana aphid *Pentalonia nigronervosa* (De Clerck et al. 2015). The elimination of *Wolbachia* consistently led to the death of aphids and the study of metabolic pathways has revealed that *Wolbachia* and *Buchnera* are working together to produce lysin and riboflavin, suggesting a mutualistic role (De Clerck et al. 2015).

Phenotypic effects of aphid facultative symbionts

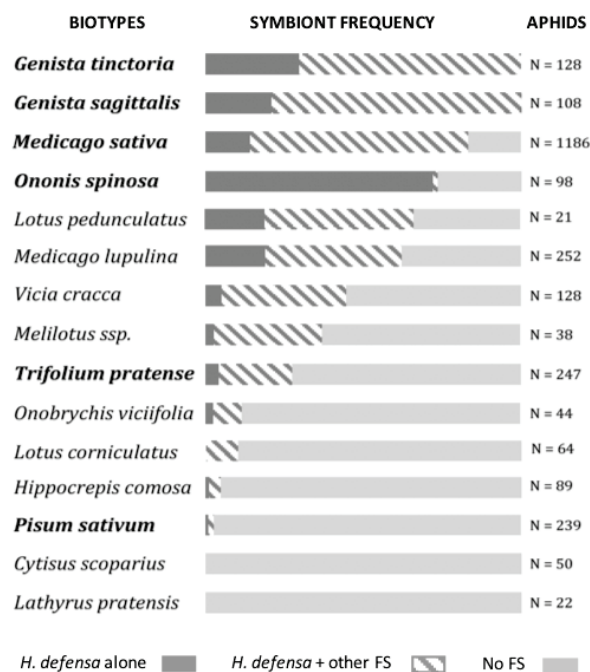
Adaptation to plant: plant biotypes

Plants have morphological and chemical defenses that impose considerable stress on aphids, which directly leads to decreased survival and inhibition of growth and reproduction. Drought, poor soil quality and herbivory can also profoundly affect the chemistry and development of the whole plant, which indirectly affect aphid health and population growth. Symbionts can in turn affect the ability of their hosts to use particular plant species. The pea aphid can feed not only on peas, but on a multitude of species of the Fabaceae family (legumes). However, all pea aphids are able to reproduce on a universal host (the bean *Vicia faba*) in the laboratory. Within the same insect species, remarkable variations in plant utilization have been frequently documented, and ecologically and genetically distinct populations are referred to as “biotype”, “host race” or “ecotype” (Gould 1983; Futuyma and Peterson 1985). Based on the performance of the pea aphid, at least 15 pea aphid races or biotypes specialized on different legume (Fabaceae) host plants have been described in western Europe (Peccoud et al. 2009, 2015). Genetic and phylogenetic analyses have revealed that some biotypes frequently exchange

genetic material, while others are reproductively isolated and may almost represent new species (Nouhaud et al. 2018).

It is interesting to note that the infection and distribution of facultative symbionts vary considerably with biotypes (Peccoud et al. 2015). For example, *H. defensa* is particularly associated with pea aphids feeding on Ononis, Genista, Lotus or Medicago in France (Table 3). In turn, *R. insecticola* and *S. symbiotica* were more commonly associated with pea aphids collected on Trifolium and Cytisus, respectively. A field study on alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*) and hairy vetch (*Vicia villosa*) in North America reported that the majority of aphids (mean = 74.2%) were infected with at least one facultative symbiont; *H. defensa* was found more often associated with aphids on alfalfa, *Regiella* with those on clover, while *Serratia* and *Regiella* were found at the same frequency on aphids on hairy vetch (Russell et al. 2013). Field studies also indicate the presence of multiple aphid-associated symbiont strains on a plant species, varying infection levels for seven species of common symbionts, and the frequent occurrence of coinfection by several species of symbionts. There are also geographical differences in the distribution of symbionts. For example, *H. defensa* is absent from pea aphid populations from Asia, Australia and South America, but is common in populations in Europe and North America.

Table 3: Distribution of *Hamiltonella defensa* among populations of aphids of 15 different biotypes in France (From Leclair 2016b)



How secondary symbionts influence the use of the host plant in pea aphid remains unclear. Phylogenetic analysis suggests that acquisition of symbionts often accompanies aphid host change, but it is not known whether this is related to host use or other ecological factors correlated with the transition to a new host. Indeed, Tsuchida et al. (2004) found that the removal of *Regiella* reduced the capacity of a pea aphid clone to feed on clover, while introduction of this same *Regiella* in a naive aphid host (*Megoura crassicauda*) improved its performance on the same plant (Tsuchida et al. 2004).

In addition to plant specialization, this genetic divergence is associated with other phenotypic differences. Pea aphids present a large repertoire of defensive strategies, including morphological, social, chemical and behavioral responses (e.g. kicking, dropping from plants) to an enemy, which are beneficial since they reduce the risk of aggression but could also be costly (Gross 1993; Guerrieri and Digilio 2008; Francke et al. 2008; Saberski et al. 2016; Bilska et al. 2018). When attacked by predators, aphids release an alarm pheromone, (E)- β -farnesene (EBF), by their cornicles, which alert conspecifics nearby on the presence of predators (Bowers et al. 1972). Exposure to this compound induces aphid escape behaviors such as dropping from the plant, but also increases the proportion of offspring that develop into dispersing winged morph (Kunert et al. 2005). *A. pisum* populations specialized on pea, clover and alfalfa showed varying degrees of defense in response to the aphid alarm pheromone, (E)- β -farnesene, (Kuner et al. 2010), which resulted in variable responses to an arthropod enemy (i.e. predators and parasitoids). Since EBF synthesis is related to the genome of aphids and not to the host plant or the primary symbiont (Bowers et al. 1972; Vandermoten et al. 2012), an effect of the secondary symbiont cannot be excluded. In addition, pea aphid biotypes showed remarkable variability in their dropping response to simulated breath of mammalian herbivores, revealing further phenotypic divergence (Ben-Ari et al. 2019). It seems that the palatability of plants for mammals promotes behavioral divergence between biotypes, reinforcing diversification through ecological divergence.

Conferred resistance to natural enemies

Predators

Symbionts may be useful or harmful for the defense of aphids against predators. The presence of *S. symbiotica* or *H. defensa* protects their hosts against a major predator of aphids, ladybug *Hippodamia convergens*, by altering the predator fitness (Costopoulos et al. 2014). Ladybug larvae feeding on pea aphids with these facultative symbionts had significantly reduced survival,

from egg hatching to pupation, and therefore a reduced emergence rate of adults. This, in turn, may select lower feeding rates on aphids with these facultative symbionts or reduce the number of reproductive adult predators around aphid colonies. Conversely *S. avenae* infected with *R. insecticola* was more predated by *Hippodamia variegata* (Coccinellidae) regardless of the host plant used (wheat or barley) but showed a positive effect on reproductive performance on wheat (Ramírez-Cáceres et al. 2019).

Body color is ecologically important to animals as it can be involved in mimicry, aposematism and crypsis (Ruxton et al. 2004), as well as species recognition and sexual selection (Leonard and Córdoba-Aguilar 2010). In natural populations of pea aphid, red and green morphs generally coexist (Moran and Jarvik 2010) and generally the red morph is dominant over green forms; Interestingly, predators preferentially exploit either red- or green-colored aphids. However, when infected with *Rickettsiella viridis*, red pea aphid larvae become green in adulthood due to the production of polycyclic quinone green pigments (Nikoh et al. 2018). Aphids become even darker green when co-infected with *H. defensa*. While ladybugs (*Coccinella septempunctata*) consumed more green than red morphs, the green forms infected by *R. viridis* are less consumed (Polin et al. 2015). To explain this behavior, it has been suggested that the presence of *R. viridis* may decrease the nutritional value of the host for ladybugs. However, aphids co-infected with *R. viridis* and *H. defensa* were more susceptible to predation suggesting an ecological cost associated with multiple infections (Polin et al. 2015).

Parasitoids

Parasitoids are species in which adults are free living individuals, but larvae develop by feeding in or on the bodies of other arthropods, usually insects (its hosts); the host may be eggs, larvae or adults that are generally killed at the end of the interaction (Godfray 1994; Quicke 2015). Parasitoids are considered in the middle of the "predator–parasite" spectrum because their mode of feeding change during their life cycle. Hymenoptera are by far the most parasitoid species-rich order of insects (La Salle and Gauld 1992). The primary parasitoids of aphids are wasps found in two taxa: the sub-family Aphidiinae (Hymenoptera: Braconidae) and the genus Aphelinus (Hymenoptera: Aphelinidae) (Sanchis et al. 2001; Kavallieratos et al. 2013). The subfamily Aphidiinae includes more than 400 described species worldwide. There are substantial differences in the biology of aphelinids and aphidiines, particularly the fact that eggs of aphidiines produce teratocytes (cells derived from egg membranes, which circulate in the host haemocoel and influence host physiology) which have never been recorded in Aphelinidae (Strand 2014). Teratocyte secretion products have been shown to reduce the growth of host

tissues (due to the secretion of proteins by teratocytes that reduce activity of the host juvenile hormone esterase) or to damage the tissues of the host (Strand 2014). These effects can release nutrients into the hemocoel, which promotes the growth of wasp larvae that feed primarily on hemolymph.

Female parasitoid wasps lay eggs preferentially in the larvae of their aphid host (Fig. 6). The parasitoid egg then hatches, and its larva develops by eating host tissues. During the L2/L3 period, the cuticle of the parasitized aphid hardens and dries, leaving an exoskeleton called mummy. At this stage, the aphid host is dead, killed by the parasitoid that consumes all the remaining tissues before pupation. The adult parasitoid emerges by cutting a hole in the mummy. Upon emergence, the female parasitoid mates and starts searching for aphid hosts.

Among natural enemies, parasitoids are considered to be the most effective agents of biological control because of their better reproduction, rapid growth, ease of rearing and positive response to increasing population density of aphids (Boivin et al. 2012; Yang et al. 2012).

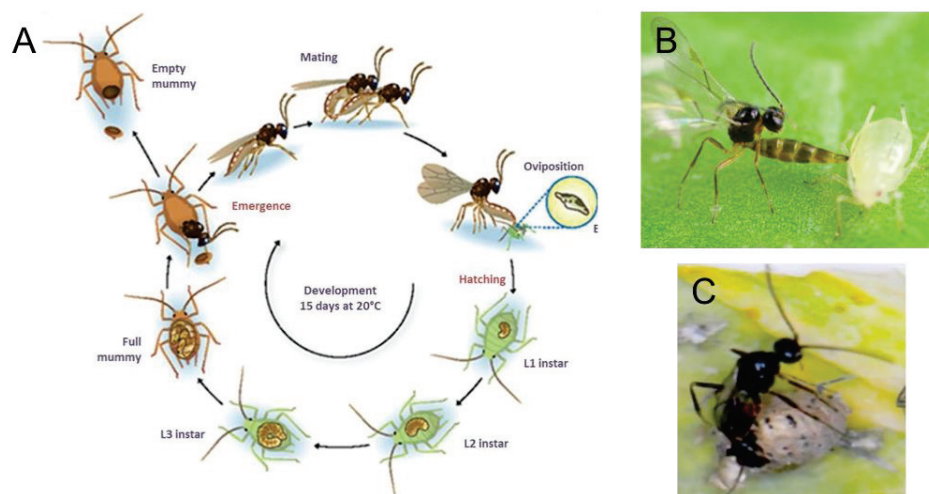


Fig. 6. Aphid parasitoid life cycle. A) complete aphid parasitoid life cycle starts with the female parasitoid oviposition (shown in B), then the egg hatches and develops at the expense of the aphid host until the host reaches its second-third instar. Then the aphid mummifies and dies, and the parasitoid completes its development before emerging by cutting a hole in the mummy (C). A, Modified from Chaubet (INRA Encyclop'aphid); B, GreenMethods.com; C, www.youtube.com/watch?v=c5ZkZw9-oAU.

Hamiltonella defensa

The protective role of a symbiotic bacterium was first detected in the interaction between *H. defensa*-infected pea aphids and the braconid parasitoid *Aphidius ervi* (Oliver et al. 2003). In natural populations, resistance to this parasite varies among clones (Ferrari et al. 2001; Dion 2011b) and is strongly associated with the presence of the bacterium (Ferrari et al. 2004). The conferred resistance to *A. ervi* was strongly correlated with resistance to the specialist parasitoid species *Aphidius eadyi* (Ferrari et al. 2004). When several isolates of *H. defensa* were

transferred into the same genetic background of *A. pisum*, from 19% to nearly 100% resistance to *A. ervi* was conferred, suggesting that it is the symbiont isolate that determines the level of resistance, not the aphid genotype (Oliver et al. 2005). Interestingly, in the cowpea aphid *Aphis craccivora*, *H. defensa* protects only against certain parasitoid species, completely suppressing the parasitism success of two *Binodoxys* species, but having no effect on *Aphidius colemani* and *Lysiphlebus orientalis* two other aphidiine braconids (Asplen et al. 2014). These findings, together with results from *A. pisum*, indicate that host protection may be a common phenotype associated with *H. defensa*, although strains have been identified in both the grain aphid, *Sitobion avenae* (Łukasik et al. 2013b), and possibly *A. pisum* that do not confer host protection. In addition, Cayetano and Vorburger (2013), using the black bean aphid *Aphis fabae*, showed that *H. defensa* has a strong capacity of protection against *Lysiphlebus fabarum* and *A. colemani*, but not other parasitoid species (*Binodoxys angelicae* and *Aphelinus chaonia*), suggesting a specificity of bacterial symbionts in host-parasitoid interactions. At least in *Aphis fabae*, some isolates of *H. defensa* strongly protect against certain parasitoid genotypes, but not or only weakly against others, leading to strong genotype-by-genotype interactions between parasitoids and defensive symbionts of hosts (Rouchet and Vorburger 2012).

Conditions may also modulate the protective phenotype of *H. defensa*. In a field experiment, Rothacher et al. (2016) showed that a defensive laboratory strain of *H. defensa* could help black bean aphids (*Aphis fabae*) to resist against wasps, while Lenhart and White (2017) showed that a defensive *H. defensa* in laboratory would lose its ability to confer resistance to *A. craccivora* under natural condition.

APSE

Bacteria can be infected by bacteriophages that may carry virulence factors in the case of pathogenic bacteria. *H. defensa* is frequently infected with a lysogenic lamdoid bacteriophage called *A. pisum* secondary endosymbiont (APSE) and it confers the protection phenotype against parasitoid wasps only when infected by the phage (Oliver et al. 2009). It has been shown that different strains *H. defensa* that provide different resistance to parasitoids are associated with different types of bacteriophages (so far named APSE-1 to APSE-8) encoding different toxins directed to eukaryotic cells (Degnan and Moran 2008a; Oliver et al. 2009; Oliver and Higash 2019).

All of the APSE types can be lost from *H. defensa* in laboratory aphid clones; once this happens, aphids immediately lose their ability to resist parasitoid wasps (Oliver et al. 2009). *H. defensa* carrying APSE2 and APSE8 that encode similar alleles of the putative cytolethal distending

toxin *cdtB* (but are phylogenetically distinct) show only moderate levels of protection (Oliver et al. 2003) whereas those infected with APSE3, which encodes a YD-repeat putative toxin (YDp), receive high levels of protection (over 80% wasp mortality) (Oliver et al. 2005). In aphids infected with *Hd*-APSE3, wasps rarely develop beyond the embryonic stage, whereas in those infected with *Hd*-APSE2 or *Hd*-APSE8, wasps often die in the larval stage >72hr after parasitism (Fig. 7) (Martinez et al. 2014). Earlier wasp mortality was suggested to be more beneficial to aphids since the wasp consumes fewer resources.

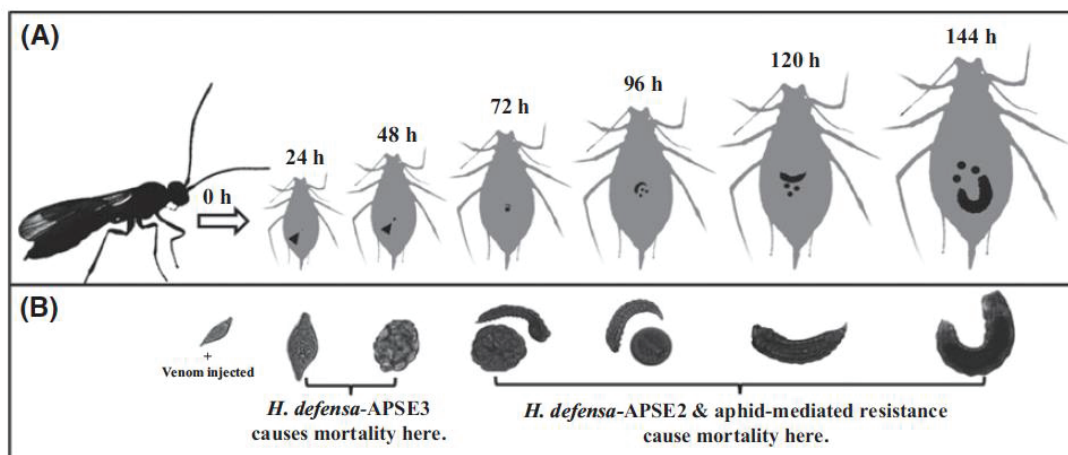


Fig. 7. Timing of *Aphidius ervi* development in aphid and *H. defensa* APSE protection. (A) Relative sizes of a free-living, adult female *Aphidius ervi* wasp and of its internally developing progeny (egg, morula and larva) compared with a parasitized 72 h old (2nd instar). (B) Enlargement of wasp developmental stages and timing of mortality observed in APSE2- and APSE3- *H. defensa*-infected aphids. 0 h: female wasp oviposition. 24–48 h: enlargement of egg and development in morula. 72 h: emergence of the wasp larva from morula and dissociation of teratocytes. 96–144 h: larval and teratocytes growth. Egg and morula are indicated by arrowheads at 24–48 h. (from Martinez et al. 2014)

One of the most striking features of *H. defensa*-mediated defense is the tremendous variation in the protective phenotype observed depending on the aphid genotype, *H. defensa*/APSE strain, and wasp genotype (see Table 4). For example, in the grain aphid *Sitobion avenae*, APSE-carrying *H. defensa* did not confer resistance to parasitism (Łukasik et al. 2013b). These non-protective *H. defensa* strains are presumably maintained in natural populations because they can provide different benefits, such as thermal protection (Russell and Moran 2005) or defense against other enemies.

Table 4. Five aphid systems in which *H. defensa* confers variable protection. Protection (P) or absence of protection (NP) against braconid (dark gray box) or aphelinid (light gray) parasitoids in different aphid species and in relation to infection with the APSE phage (from Oliver and Higashi 2019).

Aphids	Parasitoids	APSE type	P or NP
<i>Acyrtosiphon pisum</i>	<i>Aphidius ervi</i>	APSE1-like	P
		APSE 2	P
		APSE 3	P
	<i>Praon pequodorum</i>	APSE 8	NP
		APSE 3	NP
		APSE 8	NP
		<i>Aphelinus abdominalis</i>	Unknown
	<i>Aphelinus atriplicis</i>	APSE 3	NP
<i>Aphis craccivora</i>	<i>Binodoxys communis</i>	APSE 4	P
	<i>Binodoxys koreanus</i>	APSE 4	P
	<i>Lysiphlebus orientalis</i>	APSE 4	NP
	<i>Aphidius colemani</i>	APSE 4	NP
	<i>Lysiphlebus testaceipes</i>	Unknown	NP
	<i>Aphelinus atriplicis</i>	APSE 4	NP
	<i>Aphelinus glycinis</i>	APSE 4	NP
<i>Aphis fabae</i>	<i>Lysiphlebus fabarum</i>	APSE 3	P
		APSE 6	P
	<i>Aphidius colemani</i>	Unknown	NP
	<i>Binodoxys angelicae</i>	Unknown	NP
	<i>Aphelinus chaonia</i>	Unknown	NP
<i>Rhopalosiphum padi</i>	<i>Aphidius colemani</i>	Unknown	P
<i>Sitobion avenae</i>	<i>Aphidius ervi</i>	Unknown	NP
	<i>Ephedrus plagiator</i>	Unknown	NP

Parasitoids protection by other symbionts

While Oliver et al. (2003) suggested that *R. insecticola* could not protect pea aphids against parasitoid wasps, Vorburger et al (2009a) and Luo et al. (2017a) showed that the presence of some strains of *R. insecticola* could help *Myzus persicae* and *S. avenae* to resist to these enemies (see also further). In addition, several publications have also shown that coinfection of *H. defensa* with PAXS (*Fukatsua symbiotica*) in a pea aphid clone could confer higher protection against *A. ervi* than *H. defensa* alone (Guay et al. 2009; Heyworth and Ferrari 2015, 2016). A link between the abundance of aphids co-infected with *H. defensa* and PAXS aphids and parasitoid pressure has been observed, particularly in field conditions, suggesting that hosting this symbiont combination is an advantage (Leclair 2016b).

Spiroplasma are helical, cell wall-less bacteria that belong to an ancient lineage of host-associated Mollicutes. They are broadly distributed among invertebrate hosts, often crustaceans, spiders and insects and are estimated to be present in about 7% of terrestrial arthropods (Cisak et al. 2015; Cacciola et al. 2017; Ballinger and Perlman 2019). *Spiroplasma* endosymbionts are

maternally transmitted and can kill infected sons, resulting in the production of female-biased broods (Cisak et al. 2015; Cacciola et al. 2017; Ballinger and Perlman 2019). Some of these strains can also protect *Drosophila* species against a range of endoparasitoid wasps. In *D. hydei*, the native strain Hy1 protects flies against *Leptopilina heterotoma*, although survivors of wasps' attack have reduced fertility (Xie et al. 2015). Similarly, in *D. melanogaster*, the MSRO strain protects flies attacked by *Leptopilina bouvardi* (Xie et al. 2015; Paredes et al. 2016) and a strain (sNeo) that infects *D. neotestacea* appears to protect against parasitic nematodes (for review see Ballinger and Perlman 2019). In experimental populations, *Spiroplasma* spreads in the presence of nematodes, but decreases in frequency in their absence, suggesting a cost to the host. Hamilton and colleagues recently identified a *Spiroplasma*-encoded toxin, a ribosome-inactivating protein (RIP), involved in defense against nematodes (Hamilton et al. 2016). Endosymbiont-mediated protection against parasitoids may be due to competition for host lipids between the symbiont and the developing parasitoid larva (Paredes et al. 2016). The effect of *Spiroplasma* infections in pea aphid was tested using 12 bacterial strains of three different clades (Mathe-Hubert et al. 2019). Virtually all strains reduced the lifespan and reproduction of aphids, and two strains had a (weak) protective effect against the parasitoid *A. ervi* whose mechanism was not explored further.

At least, and interestingly, some pea aphid clones lacking bacterial symbionts were found to have a strong endogenous resistance, by an unknown mechanism, to parasitism by *A. ervi*, indicating that this aphid uses multiple modes of defense against its predominant parasitoid (Martinez et al. 2014).

Resistance to pathogens

Fungi

Fungal pathogens are potential environmental stressors, which could induce a deadly infection in aphids. Spores of fungi can germinate in the aphid cuticle, which then produces hyphae that invade and fill cavities and eventually kill the aphid in the following days (Oliver et al. 2010). A correlation between the presence of *R. insecticola* and the resistance to the fungal pathogen *Pandora neoaphidis*, a major fungal entomopathogen of aphids, was first reported in the pea aphid (Ferrari et al. 2004) and the involvement of *R. insecticola* confirmed by a study using five artificial pea aphid lines harboring different *Ri* strains (Scarborough et al. 2005). Additionally, three other symbionts *Rickettsia*, *Spiroplasma* and *Rickettsiella* could provide resistance to pathogenic fungi, a recent finding also suggesting that PAXS could protect pea aphids from the fungal pathogen *P. neoaphidis* (Heyworth and Ferrari 2015). Host protection

may be specific for the pathogen, a report showing that *R. insecticola* can protect *A. pisum* from *Zoophthora occidentalis*, a fungal entomopathogen specific to aphids, but not from *Beauveria bassiana*, a generalist fungal pathogen of insects (Parker et al. 2013). The mechanistic basis of this resistance, and whether different symbionts use the same mechanism (either by convergence or horizontal transfer), is not yet known.

Effects on heat tolerance

Symbionts and their hosts are generally sensitive to heat; therefore, the efficacy of endosymbionts may vary across a range of temperatures (Doremus et al. 2018), and field studies suggest that the thermal sensitivity of bacterial mutualists constrains insect responses. Although thermal limits vary by species, most adults die when the temperature is above 40 °C and a minimum of 4 °C is usually required for development (Broadbent and Hollings 1951; Hullé et al. 2010). In the field populations of *A. pisum*, temperatures of 25-30 °C reduce *Buchnera* densities and the number of bacteriocytes (Ohtaka 1991; Montllor et al. 2002), resulting in poor aphid performance such as lower survival rate and fecundity, and reduced body size and weight under high temperature conditions (Turak et al. 1998; Ma et al. 2004). Many genes normally up-regulated in response to heat are constitutively highly expressed in *Buchnera* but thermal shock increases the expression of four transcriptional promoters, affecting the expression of five heat-shock genes (Wilcox et al. 2003). In laboratory and field pea aphid populations, a deletion of a single nucleotide in a heat-shock transcription promoter for *ibpA*, encoding a small heat-shock protein, was found (Dunbar et al. 2007). This mutation eliminates the transcriptional response of *ibpA* to heat stress and decreases its expression even at cool or moderate temperatures. Furthermore, following brief heat exposure as juveniles, aphids with short-allele symbionts produced little or no offspring and contained almost no *Buchnera*. Conversely, under conditions of constant freshness, aphids containing symbionts with this mutated allele reproduced earlier and had a higher reproduction. This mutation therefore has a major effect on the host fitness in a manner dependent on the thermal environment.

There is also evidence that the facultative symbionts can help the pea aphid against heat stress (Oliver et al. 2010). Burke et al (2009) showed that *S. symbiotica* protects aphids from heat stress by protecting the *Buchnera* from the effects of heat. During heat stress, many *S. symbiotica* cells lyse, releasing substantial amounts of metabolites, and it was hypothesized that these metabolites may have protective effects on aphid or *Buchnera* cells. Other studies have also found that *S. symbiotica* or *Rickettsia* could benefit the pea aphids after heat treatments on several host plants, compared to uninfected aphids (Chen et al. 2000). *H. defensa* (Russell and

Moran 2005) and PAXS (Guay et al. 2009; Heyworth and Ferrari 2015) may also provide protection against heat stress, but to a lesser extent than *S. symbiotica*. In response to heat stress, *S. symbiotica* strains derived from warmer zone aphids provided more tolerance than those from colder areas (Russell and Moran 2005) and in the field, the *S. symbiotica* infection rate in pea aphids increases significantly during the warmer seasons (Montllor et al. 2002).

Effect on Immunity

Despite its numerous pathogenic and parasitic enemies, fungi, bacteria, viruses and parasitoid wasps, the immune system of aphids is reduced relative to other insects. Annotation of different aphid genomes, including pea aphid, has shown the absence of many components central to immune function in other insects (The International Aphid Genomics Consortium 2010; Gerardo et al. 2010). Most pathogen recognition receptors PGRPs involved in bacterial recognition, the *imd* gene and many genes involved in antimicrobial peptides (AMPs) production are absent in this species. This deficiency in antibacterial response to Gram negative bacteria might have evolved as a protection of the obligate and facultative bacterial symbionts from the immune system (Gerardo et al. 2010; Burke and Moran 2011). However, other authors have proposed that facultative symbionts manipulate the host immune response to protect themselves (Gross et al. 2009; Welchman et al. 2009) and that these symbiotic microorganisms participate in host immunity (defensive symbioses) by enhancing their resistance for example against parasitoids (Kaltenpoth and Engl 2014). The adoption, tolerance, maintenance, and transmission of bacterial secondary symbionts among aphid populations are therefore important in defense strategies that evolved over time (Haine 2008). A recent study has shown that the presence of some facultative symbionts can affect the cellular immunity of pea aphids by reducing the number and modifying the proportion of the adherent hemocytes with phagocytic and encapsulation properties (Laughton et al. 2011; Schmitz et al. 2012; Laughton et al. 2016). The presence of facultative symbionts also affects the humoral immune response of aphids (Schmitz et al. in prep), but the mechanisms involved remains unknown. At last investment in defense can also have fitness costs and the increase in reproduction after a stress reported in aphids suggests occurrence of trade-offs (immunity will be more detailed on Part 2).

Effects on life parameters

Symbionts can have “direct costs” for aphids when there is a trade-off between allocating resources to symbiosis and to other functions such as reproduction or growth. Symbiosis can also negatively affect the interactions between the host and other organisms in the environment,

resulting in an ecological cost (McLean 2019). The presence of facultative symbionts was often associated with such costs in aphids. *H. defensa* reduce the fecundity of pea aphids (Simon et al. 2011) and affect their survival depending on the genotype of the host (Leclair et al. 2017). *S. symbiotica* and *Rickettsia* reduce the lifespan and offspring number of pea aphids, reduce their growth and delay their development (Koga et al. 2003; Sakurai et al. 2005; Simon et al. 2007; Łukasik et al. 2013b). The magnitude of the effects of *Rickettsia* varied with host genotypes and its effect on fecundity depends on aphid species (Sakurai et al. 2005). *R. insecticola* increases the offspring number in pea aphid on white clover, but not on vetch (Tsuchida et al. 2004). Other studies have observed negative effects of this symbiont on fecundity and development in the grain aphid, *Sitobion avenae* (Wang et al. 2016; Luo et al. 2017b). Therefore, the magnitude of *R. insecticola* effects on fecundity may depend on plants and/or aphid species. Sakurai et al. (2005) showed that *Spiroplasma* had no effect on the fecundity of 8 different aphid genotypes of two aphid species (*A. pisum* and *S. avenae*), but Fukatsu et al. (2001) and Simon et al. (2011) suggested that *Spiroplasma* may reduce the fecundity and survival rate of pea aphids. One possible explanation could be the difference between symbiont strains. In addition, *Rickettsiella* decrease the offspring number of pea aphids by reducing their reproductive or survival time (Tsuchida et al. 2014; Leclair et al. 2017). However, these fecundity variations were host genotype-dependent (Leclair et al. 2017). Lastly, PAXS may negatively impact the fecundity of the pea aphids (Heyworth and Ferrari 2015).

Endosymbionts can narrow the host range of parasitoids by reducing their ability to locate hosts through interactions with the host plants. Some parasitoids can detect specific volatiles emitted by plants to locate their prey. Aphid defensive symbionts can alter the profile of volatile compounds emitted by their feeding plants and thus reduce their attractiveness to parasitoids (Frago et al. 2017). They could also affect the fitness traits of the emerging adult parasitoid and/or modulate the outcome of competition between parasitoid species (for reviews McLean 2019; Monticelli et al. 2019).



OBJECTIVES

Numerous studies are under way to characterize the variation of symbiotic defenses, to understand the maintenance and functioning of defensive symbionts under natural conditions, to evaluate the potential for symbiont-mediated coevolution, and to explore how defensive symbioses influence biotic interactions and impact other community inhabitants. My thesis work is related to several of these different aspects, investigating from a physiological and ecological point of view the impact of bacterial symbionts of aphids on the success of parasitoids and on the immune system of their host as well as their fitness in order to establish a link between these different levels.

The first part of the thesis presents 2 studies on the host-parasitoids interaction and the role that symbionts can play. In Publication 1, based on my previous work in China and performed in collaboration, I have examined the specificity of host-parasitoid interactions in relation to a defensive bacterial symbiont. For this study, I used *Sitobion avenae* aphids infected artificially with a protective strain of *Regiella insecticola* to test the level of protection conferred to parasitoids used in biological control. Then, I participated in a study (Publication 2) to refine the classification of three aphid parasitoids using the preference-performance hypothesis (PPH) which provides useful cues to classify parasitoids in terms of degree of specialization. For this, we used 12 aphid species maintained on their host plants and for which the symbiotype was defined. We therefore also analyzed the effect of the symbiont on the success of parasitoids.

In many insects, the success of parasitoids is linked to the inhibition of the host immune response against the parasitoid egg/larva, which relies on both cellular and humoral components. This inhibition results in general from the action of venom components injected at oviposition. The second part of my thesis is dedicated to the molecular study of the effect of different symbionts on an important humoral immune component of the aphid involved in this response, the phenoloxidase. Publication 3 shows the different approaches (mass spectrometry, antibodies, molecular biology) used to analyze the amount and the activity of phenoloxidases in different aphid clones (genotype effect) and in presence of the three main symbionts *Hd*, *Ri* and *Ss* either naturally present or after artificial infection. I have also searched for a relation between the level of PO and the fitness of aphids as well as the relationship between PO levels and the response to biotic and abiotic stressors. I also used these tools to analyze the expression of PO during the larval development of the pea aphid.

RESULTS

PART 1 - PARASITOIDS-APHIDS-SYMBIONTS PROTECTION

Pesticides are dangerous for the ecosystem and some can be harmful to human health (Pimentel 1997; Gill and Garg 2014; Lewis et al. 2016) which, along with the continual increase in resistance of pests (Barres et al. 2016; Hawkins et al. 2018), increases demand for safer and more sustainable methods to protect agricultural crops, especially in the context of Integrated Pest Management (IPM). Biological control (biocontrol) is a safe and sustainable approach that exploits natural enemies such as predators, parasitic insects or pathogens to manage pests in agroecosystems. Dozens of species of predatory insects and parasitoids are reared worldwide, and in some cases these programs have proved economically competitive with other control methods (van Lenteren 2011). In 2010, at least 230 species of invertebrate natural enemies—from, ten taxonomic groups, were used in pest control worldwide. The majority of species belong to arthropods (219 out of 230 species) and 52.2% were parasitoids of the Hymenoptera order (van Lenteren 2011). Today, parasitoids are the natural enemies most used in classical biocontrol in the world.

As presented in the introduction, insect parasitoids have an immature life stage that develops on or in a single insect host, eventually killing the host, hence the value of parasitoids as natural enemies. Adult parasitoids live freely and feed on non-host resources such as nectar and/or on host hemolymph by food sting, a process called 'host-feeding' (Jervis et al. 2008; Strand and Casas 2008). Most parasitoids attack only a particular stage of the host life. The immature parasitoid develops on or in the pest, feeding on bodily fluids and organs, eventually allowing the host to pupate, and emerging from the host pupa as an adult (see Fig. 6). The life cycle of the host and parasitoid may match, or that of the host may be altered by the parasitoid to suits its own development.

Some parasitoids have one or a few related host species (thus consider specialists), while others have a wider host range (generalists) (see Table 5 for definitions). Parasitoids with more than one host species are also called oligophagous, with host species preferences of polyphagous parasitoids being more diverse. In contrast, parasitoids with high host specificity are called monophagous parasitoids, which have a high degree of ecological compatibility. Host preference is the most important behavioral characteristic in parasitoids; it is influenced by various factors such as host species, host stage, host size, host density, and intraguild predation. In addition, various biotic and abiotic factors are involved in the determination of both host

preferences and host specificity of parasitoids in the host selection process, which are divided into chemical and physical parameters. Examples include plant volatiles, host habitat, host odors, host movements, sound, and environment color. In all cases, host preference and specificity may affect the growth rate of the host population and the dynamics of the host-parasitoid population. To be successful in biological control programs, it is necessary to understand the physiology of the host and the preferences and specificity of the parasitoid to predict the outcomes in the field.

Table 5. Some definitions used to describe parasitoids life parameters.

Primary parasitoid	Species that develops on non-parasitized hosts
Hyperparasitoid (or secondary parasitoid)	Parasitoid species that develops in another parasitoid (a parasitoid of a parasitoid). There can be several levels of hyperparasitism.
Endoparasitoid	Parasitoid that develops inside the body of the host
Ectoparasitoid	Parasitoid that develops outside the body of the host (they feed by inserting the buccal parts through the integument of the host)
Multiple parasitism	Situation in which more than one species of parasitoid are found within or on a single host.
Superparasitism*	Several individuals of a species of parasitoid can be found in a host

*When superparasitism occurs with solitary endoparasites, mutual destruction or physiological suppression of larvae or surplus eggs may result in survival of a single dominant individual. In some cases, however, the host dies prematurely, before the surplus is eliminated, and therefore all the protagonists die.

Specificity of a bacterial symbiont in *S. avenae*-parasitoids interactions

Sitobion avenae

Aphids are one of the most challenging insect species to manage because of the rapid population growth rate, direct feeding effect on a range of economic crops, and indirectly pathogen transmission including many viruses (Van Emden and Harrington 2007). In China, about 23.4 million hectares of agricultural land are planted with wheat, of which 10-15 million hectares are infested with cereal aphids, resulting in a yield loss of at least 10% per year (Chinese National Agro-Tech Extension and Service Center [NATESC] database, <http://www.natesc.moa.gov.cn/>). There are four main aphid pests in China: *Sitobion avenae*, *Rhopalosiphum padi* (L.), *Schizaphis graminum* (Rondani), and *Acyrtosiphon dirhodum* Walker (Lu and Gao 2009). The English grain aphid, *Sitobion avenae* (Fab.) (Fig. 8), is the dominant cereal aphid species, causing the most damage at the wheat filling stage (Zhang et al. 2009).



Fig. 8. The English grain aphid *Sitobion avenae*. *S. avenae* is elongated in shape and has a length of 2 to 3 mm. Its color can vary from green to red or yellow and it has black cornicles. This aphid colonizes the leaf blade of the upper leaves, then develops mainly on the ears as soon as they emerge. (Image from encyclop'aphid).

The aphid species *Sitobion avenae* (Sternorrhyncha: Aphididae) is found on many Poaceae species worldwide (Fig. 9) and is one of the most potentially damaging pests for cereals (Wanf et al. 2016). It is a major pest of wheat (*Triticum aestivum*) and a secondary pest of rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and some other cereals. *S. avenae* causes direct damage by feeding on fruits, leaves, stalks and ears, and indirect damage by excreting honeydew and transmitting viruses. *S. avenae* primarily affects grain yields by removing plant nutrients and reducing photosynthesis via honeydew accumulations. Honeydew also promotes the growth of secondary fungal pathogens and may account for additional loss.

Wheat yields can be reduced by 20-30% during outbreaks (Kolbe and Linke 1974). For example, a 3-year German field study reported yield reductions of 11.5-43.4%, resulting from early infestation at the time of ear emergence (Hinz and Daebeler 1976). In a field study in China, a reduction in wheat yield up to 14% was observed following an early infestation, while the effect on yield loss would decrease with subsequent infestations (Liu et al. 1986). Infestations

occurring during grain ripening generally do not cause significant yield losses but may reduce the quality of the flour (Wratten 1979).

S. avenae is also an important vector of barley yellow dwarf virus (BYDV). Of the six strains within the BYDV complex, *S. avenae* is a major vector of two: BYDV-MAV and BYDV-PAV (Brunt et al. 1996). Depending on the region, *S. avenae* could be the main vector of BYDV primary infection in cereals in autumn or may be involved in the spread of the virus in spring (Leclercq et al. 1995).



Fig 9: *S. avenae* world distribution. Red dots indicate the presence of *S. avenae*. (From CABI).

Survey in China reveals several *S. avenae* symbiotypes

Evidence has shown that at least in some crops such as cereals, parasitoids may be the most important agents for reducing pests (Schmidt et al. 2003). In this context, because endosymbionts can confer improved defenses against natural enemies, particularly parasitoids, to host aphids (Oliver et al. 2010), the efficacy of biological control with such parasitoids could be compromised (Oliver et al. 2005; Käch et al. 2017).

As part of my master's thesis, I participated in the study of *Sitobion avenae* from six different locations in China and in their screening for the presence of endosymbionts (Luo et al. 2016). An unexpected result was that *S. symbiotica* and *R. insecticola* could be detected in all aphid populations, whereas *H. defensa* was detected in three locations only (Table 6). This result, consistent with previous data, indicated that all *S. avenae* clones from different other locations in China were also naturally infected by *R. insecticola* (Wang et al. 2016).

No.	Locality	Longitude/ latitude	Infection rate		
			<i>R. insecticola</i>	<i>H. defensa</i>	<i>S. symbiotica</i>
1	Yangling, Shaanxi	34.36°N, 108.72°E	1	0.30 (3/10; 2/10; 4/10)	1
2	Wudingguan, Shaanxi	32.33°N, 118.31°E	1		1
3	Chuzhou, Anhui	32.83°N, 106.25°E	1	0.66 (7/10; 6/10; 7/10)	1
4	Tigu, Shanxi	34.36°N, 110.15°E	1		1
5	Zhengzhou, Henan	34.46°N, 113.40°E	1		1
6	Shihezi, Xinjiang	44.18°N, 86.00°E	1	0.17 (2/10; 1/10; 2/10)	1

Table 6. Sample locations of the different geographic populations used for the study and the infection rates of *R. insecticola*, *H. defensa* and *S. symbiotica*. The numbers in parentheses show the infection frequency of endosymbionts; the notation n/10 indicates that n (number) individuals were infected of 10 individuals (1=10/10); a blank entry means 'not detected' in that population. From (Luo et al. 2016).

Parasitoid species composition in wheat fields in China

More than 630 species of aphid parasitoids are recorded in Aphidiinae, of which 132 species are found in China. Among these species, *Aphidius gifuensis* and *Aphidius colemani* are the two primary parasitoid wasps of the green peach aphid, *Myzus persicae*, in China. In practice, *A. gifuensis* has managed to successfully control aphid populations, and the rate of parasitism could reach 89% in the field (Gu et al. 2015; Wang et al. 2019).

In a study of wheat fields in northern and northwestern China, 11 primary parasitoid species and 15 hyperparasitoid species were identified from the collected mummies (Yan et al. 2016; Yang et al. 2017). Among them, three species, *Aphidius uzbekistanicus* Luzhetskii, *Aphidius gifuensis*, *Aphidius ervi* were the most abundant primary parasitoids. The second-most abundant species were *A. ervi*. The hyperparasitoid guild was more diverse, consisting of 14 species dominated by *Pachyneuron aphidis* Bouché, accounting for about 35% of the total, and *Asaphes suspensus* Nees. The seasonal dynamics of parasitoids showed that primary parasitoids appeared during the early growing season and hyperparasitoids at the end of the growing season. More specific studies have shown that *A. asychis* and *A. gifuensis* are the two dominant primary parasitoid species in suppressing *Sitobion avenae* population growth (Liu et al. 2009; Wang et al. 2011).

R. insecticola conferred parasitoid protection to *S. avenae*

While in pea aphid *H. defensa* confers resistance to wasps (Oliver and Higashi 2019), studies showed that *R. insecticola* is mainly involved in protection to some fungal pathogens (Parker et al. 2013). In studies that investigated the relationship between *R. insecticola* and parasitism, the results are mixed. In *A. pisum* (Oliver et al. 2003) and in *Aphis fabae* (Vorburger et al. 2009b), *R. insecticola* did not provide any protection against parasitoids. However, von Burg et al. (2008) using 17 clones of the peach potato aphid, *Myzus persicae*, for which life-history traits were available, found significant clonal variation for susceptibility to two of their common

parasitoids: *Aphidius colemani* and *Diaeretiella rapae*. An Australian clone, the only one harboring the facultative *R. insecticola* (named strain 5.15), was entirely resistant to both parasitoids. To test whether the endosymbiont did confer resistance, $Ri^{(5.15)}$ was transferred to two other clones of *M. persicae* and one clone of *Aphis fabae*, a different aphid species. The exposure of these lines to the parasitoid *A. colemani* clearly showed that $Ri^{(5.15)}$, unlike other *Ri* strains, provides strong protection against parasitic wasps (Vorburger et al. 2009a). Negative effects of $Ri^{(5.15)}$ on host survival and lifetime reproduction were limited and frequently non-significant, and these effects were comparable or in one case weaker than those of *R. insecticola* strains that are not protective against parasitoid wasps (Jamin and Vorburger 2019). At last, this research group sequenced the genome of $Ri^{(5.15)}$ and compared its gene repertoire to that of the nonprotective strain $Ri^{(LSR1)}$; revealing striking differences in gene sets related to eukaryote pathogenicity (Hansen et al. 2012). $Ri^{(5.15)}$ encoded five categories of missing or inactivated pathogenicity factors in $Ri^{(LSR1)}$ including the type 1 Secretion System and its secreted RTX toxins and an intact SPI-1 Type 3 Secretion System and its effectors. These pathogenicity factors and translocation systems are thought to play collectively play a key role in the virulence of endosymbiont against parasitoids. These results suggest that the effects of *R. insecticola* on parasitism resistance may differ among bacterial strains.

Therefore, in order to know whether *R. insecticola* strains may or may not play a role in the protection of *S. avenae* against parasitoids, we isolated different aphid individuals harboring *R. insecticola* and used them as donors to create by microinjection new aphid lines derived from clones lacking secondary symbionts (Table 7) (Luo et al 2017b).

Table 7. Production of *R. insecticola* infected *S. avenae* clones. Clones without *R. insecticola* were collected in Neixiang and in Linyi (China). *R. insecticola* donor clones were collected in Yangpingguan and named Yangpingguan 1 and Yangpingguan 2. Each clone was derived from a single parthenogenetic *S. avenae* female. Body fluids from the Yangpingguan clones 1 and 2 were transferred to the Linyi clone and the Neixiang clone, respectively, resulting in creation of four new clones.

Clones	Genotype	<i>R. insecticola</i> donor
Linyi-1	Linyi	Yangpingguan 1
Linyi-2		Yangpingguan 2
Linyi-NA		Control
Neixiang-1	Neixiang	Yangpingguan 1
Neixiang-2		Yangpingguan 2
Neixiang-NA		Control

We characterized the life parameters of these created strains and demonstrated that infection with *R. insecticola* had a negative effect on the natural development of *S. avenae* clones, then

on the potential growth of the population, and that levels of impact differed according to the genetic background (Luo et al. 2017b).

In an initial study using only Neixiang clones, we found that infected aphids were more likely to be parasitized by the wasp *Aphelinus asychis* (Aphelinidae), a common and important parasitoid of aphids used in biological control (Wang et al. 2016). After parasitism, mummies were 4.7- and 4.9-fold more abundant in Neixiang-1 and Neixiang-2 infected with *R. insecticola* respectively, than in controls (Luo et al. 2017a; Fig. 10). However, the emergence rate was similar, suggesting an increased mortality of parasitoid larvae in the presence of *Ri*. In addition, the weight of parasitoids emerging from *Ri*-infected aphids was reduced by about 40% compared to the control.

Although unexpected and somewhat disturbing these results indicate that *R. insecticola* may be involved in the interaction between *S. avenae* and one of its main parasitoid wasps. However, these results could be due to a specific (aphid clone x symbiont strain) genotype-genotype interaction and/or depend on the parasitoid species chosen.

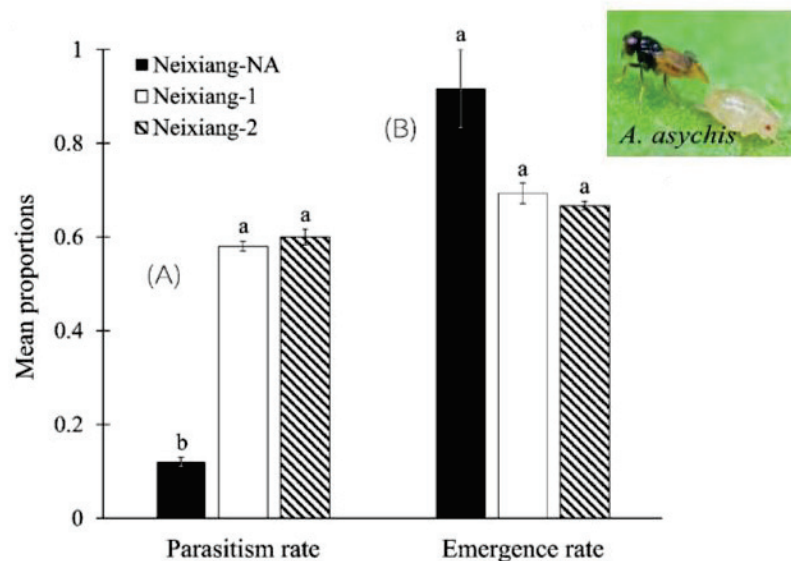


Fig. 10. Comparisons of the parasitic rate (A) and emergence rate (B) of *A. asychis* among *R. insecticola*-infected *S. avenae* (Neixiang-1 and Neixiang-2) and uninfected clones (Neixiang-NA). Each value is the mean \pm SEM. (Different letters above bars within each panel indicate significant differences at $P < 0.05$).

Therefore, in order to check whether or not the *R. insecticola* strain has a general protective effect, the protection against the species *Aphelinus asychis* and a second parasitoid species, *Aphidius gifuensis* (Braconidae, used for biological control of *Myzus persicae* and *S. avenae*) was studied on a different genotype of *S. avenae* (Linyi clones) ([Publication 1](#)).

Publication 1: Specificity of a bacterial symbiont in host-parasitoids interactions

The results showed that for the new *S. avenae* Linyi genotype, the lines artificially inoculated with the *R. insecticola* suffer a higher parasitism rate by *A. asychis* compared to the uninfected line, and the corresponding emergence rate was lower due to higher mortality during development, which is consistent with the previous study. With *A. gifuensis*, no difference in the parasitism rate and emergence rate was observed between the infected lines and the uninfected control line, thus the increase wasp susceptibility induced by *R. insecticola* is wasp species-dependent. However, we found an adverse effect of *R. insecticola* on the weight of the emerging wasps for the two species suggesting a metabolic cost.

We also examined the life-history traits of surviving aphids from the wasps' attack. *R. insecticola* infected aphids having survived *A. asychis* attack had a strong decrease in fecundity (~1.63 fold) and survival rate (~1.59- fold) compared to uninfected ones; Infected aphids having survived *A. gifuensis* attack had no difference in fecundity and a slight, but significant 1.1-fold survival rate. The higher mortality of *A. asychis* larva in the presence of *R. insecticola* is thus possibly linked to an adverse effect on the aphid fitness.

Publication 1

An increased risk on aphid parasitism mediated by facultative symbiont *Regiella insecticola*

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An increased risk on aphid parasitism mediated by facultative symbiont *Regiella insecticola*

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Abstract

Previous data showed that the parasitoid wasp *Aphelinus asychis* was more successful on *Sitobion avenae* clones artificially infected with different strains of *Regiella insecticola* compared to the same clones lacking facultative symbionts. To test whether this resulted from a specific interaction between the aphid and symbiont genotype, we used new clonal lines from a different genotype of *S. avenae* (Linyi) infected with the same *R. insecticola* strains. The parasitism rate of *A. asychis* was higher on the two Linyi lines infected with *R. insecticola* than on the uninfected control line, while the emergence rate was lower due to higher mortality during development. However, a different wasp species, *Aphidius gifuensis*, showed no difference in the parasitism rate and emergence rate between the three Linyi lines, suggesting that the increase in parasitism rate could be wasp species-dependent. The mortality of *A. asychis* during development in the presence of *R. insecticola* may be linked to i) a direct toxic or metabolic effect since the weight of the emerged wasps was also reduced, and ii) a general effect on the aphid fitness since the survival and fecundity of infected aphids having survived *A. asychis* attack are clearly reduced compared to the control, while those surviving *A. gifuensis* attack had only a slight increase in their survival rate. Our data therefore enriched the panel of phenotypic effects that *R. insecticola* could contribute to the aphid and emphasized the potential implications of symbionts on biological pest control.

Keywords: *Sitobion avenae*; parasitoid wasp; *Regiella insecticola*; aphid genotype; symbiont strains

Key messages:

- A growing body of evidence suggests endosymbionts infecting insects may disrupt biological control of pests.
- The grain aphid, *Sitobion avenae*, is a globally important pest, but knowledge of its symbionts, which may affect pest control, is limited.
- Here, we show that *Aphelinus asychis* was more successful in parasitizing *Sitobion avenae* infected with the symbiont *Regiella insecticola*.

Introduction

Many animals, including several insects, live in symbiosis with vertically transmitted bacteria that greatly influence the biology and ecology of their host by conferring it an extended phenotypic plasticity (Margulis and Chapman 1998; Moran 2007; Hurst 2017; Hunter 2018). A widely studied example in recent years is the relationship between the pea aphid (*Acyrtosiphon pisum*) and its heritable symbionts, which is emerging as a model system to explore how the host phenotype is modulated (Buchner 1965; Douglas 1989; Oliver et al. 2010; Schmitz et al. 2012; Łukasik et al. 2013; Aspölen et al. 2014; Rothacher et al. 2016; Skaljac 2016; Vorburger et al. 2018).

Aphids harbor an obligatory bacterial symbiont, *Buchnera aphidicola*, in specific cells called bacteriocytes. *B. aphidicola* provides the host with essential amino acids that it does not find in the phloem sap and cannot synthesize (Buchner 1965; Skaljac 2016). In addition, aphids can be infected by one or more other facultative (secondary) symbionts found either in secondary bacteriocytes, free in the hemolymph and/or inside the immune cells (Baumann et al. 1995; Brady et al. 2014; Fukatsu et al. 2000; Oliver et al. 2010; Schmitz et al. 2012). Among the nine facultative symbionts described in pea aphid individuals collected in the field, the most frequently identified are *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica*, while others are rare or found in few individuals in some surveys (Oliver et al. 2014; Zytynska and Weisser 2016; Guo et al. 2017; Cariou et al. 2018). These facultative symbionts are not present in each individual aphid and are not necessary for survival and normal reproduction of the host, but they largely influence host phenotypes such as color, plant specialization, behavior, protection against pathogens and natural enemies or tolerance to heat. In return, some of these symbionts also have a negative impact on the fitness of the host (Tsuchida et al. 2004; Desneux et al. 2009a; Simon et al. 2011; Henry et al. 2013; Gauthier et al. 2015; Giron et al. 2017; Monticelli et al. 2019a). In addition, the aphid phenotype observed in the presence of a symbiont may vary according to the aphid species, the genotype of the aphid and the symbiont genotype (Hafer and Vorburger 2019). For instance, *H. defensa* protects different species of aphids against certain parasitoid wasps such as *Aphidius ervi* and *Lysiphlebus fabarum* (Oliver et al. 2003; Vorburger et al. 2013; Oliver and Higashi 2019). This protection is linked to the presence of toxin-producing bacteriophages named APSE (*A. pisum* secondary endosymbiont) associated with *H. defensa*, with protection levels varying according to the associated APSE strain (Oliver and Higashi 2019). *S. symbiotica* can provide pea aphids with resistance to heat possibly by a lysis mechanism releasing metabolites that protect heat-sensitive *Buchnera* symbionts (Montllor et al. 2002, Burke et al. 2009). The presence of *S. symbiotica* also increases the resistance to the *A. ervi* parasitoid wasp, the developing wasp larvae being killed as in the presence of *H. defensa* with APSE (Oliver et al. 2006), although the exact mechanism involved is not yet elucidated. *R. insecticola* infection is mainly known to enhance resistance of host aphids to pathogenic fungi (Scarborough et al. 2005; Parker et al. 2013). In the pea aphid, the natural occurrence of *R. insecticola* did not affect *A. ervi* parasitism rates (Oliver et al. 2003), but the transfer of a specific strain (*R. insecticola* 5.15) from a resistant clone of the peach-potato aphid *Myzus persicae* in non-resistant *M. persicae* or another aphid species (Von Burg et al. 2008) (*Aphis fabae*) increased their resistance to the parasitoid *Aphidius colemani* (Von Burg et al. 2008; Vorburger et al. 2010). This protective effect may be due to the presence in its genome of pathogenicity factors and translocation systems absent from other strains (Hansen et al. 2011).

In a recent survey of wheat aphid (*Sitobion avenae*) populations from different locations in China, we found that a large majority of aphids were infected by *R. insecticola* (Luo et al. 2016). Different individuals carrying *R. insecticola* were isolated from these populations and these *R. insecticola* then as donors were used to create new aphid lines by micro-injection in aphid clones free of secondary symbionts (Luo et al. 2017a). One of these clone families (Neixiang origin) was used to test whether *R. insecticola* could confer resistance to the parasitoid wasp, *Aphelinus asychis* Walker (Hymenoptera: Braconidae), a major natural enemy of aphid field populations, often used for biological control in China (Pan et al. 2014; Wang et al. 2016). The parasitism rate was increased for Neixiang clones infected with *R. insecticola* compared to the original clone devoid of facultative symbiont whereas the presence of the symbiont decreased the emergence rate (Luo et al. 2017b). Since this striking result may be due a specific aphid clone x symbiont strain genotypic interaction, we conducted similar experiments using a different genotype (Linyi) of *S. avenae* transfected with the same *R. insecticola* symbiont strains. We also examined whether the observed phenomenon extended to a different parasitoid species (*Aphidius gifuensis*) and how the presence of the symbionts could affect the fitness of aphids under parasitoid stress condition.

Material and methods

Biological materials

The different *S. avenae* clones used herein have been previously described and screened by PCR with primers specific for the presence of all the symbionts documented in the aphids (Luo et al. 2017a; Desneux et al. 2018; Monticelli et al. 2019b; see Table S1 and S2). The Linyi-NA original clonal line, devoid of facultative symbionts, was established from an individual collected at Linyi (35.05°N, 118.35°E), Shandong Province, China. Linyi-NA was used to artificially produce two clones, Linyi-1 and Linyi-2, by micro-injection of two *R. insecticola* strains from the *S. avenae* Yangpingguan 1 and 2 donor clones, respectively. Methods for distinguishing the two strains by comparing the sequences of 16S rRNAs and phylogeny followed the procedure described by Unterman et al. (1989) (Luo et al. 2017b; Luo et al. 2020; GenBank number: MN555711 and MN555712). All aphids were maintained on wheat (*Triticum aestivum* L. cv. ‘Aikang 58’) at 20°C, 75% RH and a 16:8 h light/dark cycle. Linyi-1 and -2 strains were regularly screened by PCR for the persistence of *R. insecticola* and they had been maintained in the laboratory for more than four years at the onset of the reported experiments.

The wasps *A. gifuensis* and *A. asychis* were produced as described previously (Luo et al. 2017b; Luo et al. 2020) on an aphid clone devoid of facultative symbionts, and the adult wasps maintained in separate cages (25x25x25cm) and fed with hydromel. Four-day-old female wasps assumed to be mated were used. Thirty minutes before the assay, 5 uninfected second instar aphids (Linyi-NA) in a Petri dish were exposed to the female wasps and only wasps that had stung in less than 5 min were used. The experiments were carried out under the conditions where wasps were maintained. We previously showed that injection of PBS (phosphate-buffered saline) as a control had no effect on parasitism and life-history traits (Luo et al. 2017a; Luo et al. 2020).

Parasitism and fitness assays

The basic design of the experiment was to compare the parasitism of the two parasitoid species *A. gifuensis* and *A. asychis* on the Linyi-NA aphid devoid of facultative symbionts (control condition) and the Linyi-1 and Linyi-2 clones persistently infected with the *R. insecticola* from Yangpingguan 1 and 2 donor clones, respectively. We also analyzed the impact of *R. insecticola* on fitness-related traits (reproduction, survival) of aphids having

survived the parasitoid attack and the weight of the emerged parasitoid. For each experiment, 30 adult aphids (~13 days old) were transferred on a new wheat plant and removed after 12 hours. After 4 days, 30 second instar offspring were collected and transferred to an aerated transparent container (\varnothing 6 cm and 25 cm height) with one wheat plant. After 12 hours of adaptation, 3 female wasps (either *A. gifuensis* or *A. asychis*) were introduced into the cages and remained for 6 hours. Then 4 days later, the 30 aphids were individually transferred into Petri dishes (\varnothing 9 cm) containing a fresh wheat leaf that was replaced every 2 days. The number of mummies, emerged wasps, as well as the aphid offspring number and the mortality of non-parasitized (i.e. non-mummified) aphids were recorded daily following the transfer until the last aphid died. Parasitism rate (PR) was measured by the number of mummified aphids / the number of whole treated aphids (30 aphids); emergence rate (ER) was measured by the number of parasitism success / the number of mummified aphids. The wasps were weighed within 24 hours of emergence using a high precision balance (Mettler Toledo XS3DU; Mettler-Toledo GmbH, Switzerland). A total of 36 “host line \times parasitoid” combinations were performed: three aphid lines \times two parasitoid wasps \times 6 replicates per host \times parasitoid assay.

Statistical analysis

All data were analyzed using R version 3.4.3 (www.r-project.org). We used generalized linear modelling implemented to compare the effects of the presence of the symbiont on parasitism and emergence rates; a quasibinomial error distribution (link = logit) was used, which considers any overdispersion in the data. To test the differences of aphid lines on the weight of emerged wasps and the fecundity of non-parasitized aphids, a linear mixed model effect of the ‘lmerTest’ package was used (with repetition as a random effect). Aphid survival was analyzed using the cox proportional hazards model (Cox 1972) from the survival package (Therneau 2015). The package ‘multcomp’ was used to perform multiple comparisons using the Tukey’s multiple range test.

Results

Effect of the presence of *R. insecticola* on resisting to the parasitoid

For *A. gifuensis*, the parasitism rate (PR), measured by the number of mummified aphids, and the emergence rate (ER) of parasitoids from these mummies were similar between the three lines (control and infected lines) (PR: $\chi^2 = 0.19$, $df = 2$, $P = 0.86$, Fig. 1A; ER: $\chi^2 = 0.26$, $df = 2$, $P = 0.78$, Fig. 1B) and between Linyi-1 and Linyi-2 ($P > 0.05$ for both PR and ER). For *A. asychis*, however, the presence of *R. insecticola* significantly increased the PR ($\chi^2 = 28.37$, $df = 2$, $P < 0.001$; Fig. 2A), whereas a decrease in the ER was observed ($\chi^2 = 29.04$, $df = 2$, $P < 0.001$; Fig. 2B). The rate of formation of aphid mummies increased 1.62- and 1.64- fold in Linyi-1 and Linyi-2, respectively ($P < 0.001$ and $P < 0.001$) while the emergence rate was decreased 1.70- and 1.64- fold ($P < 0.001$ and $P < 0.001$) compared to the control. There was no significant difference between Linyi-1 and Linyi-2 for either PR or ER ($P = 0.98$ and $P = 0.91$, respectively).

Effect of the presence of *R. insecticola* on the weight of emerged wasps

Compared to Linyi-NA, the weight of the emerged parasitoid wasps was significantly lower in the presence of *R. insecticola* ($F_{2, 79} = 654.40$, $P < 0.001$ for *A. gifuensis*, Fig. 3A; $F_{2, 81} = 24.78$, $P < 0.001$ for *A. asychis*, Fig. 3B) but no difference was observed between the *R. insecticola* infected lines ($P = 0.81$ and $P = 0.98$, respectively). The

weight of the *A. gifuensis* wasps emerged from Linyi-1 and Linyi-2 lines was decreased by ~1.3- fold compared to the control and ~1.1-fold for *A. asychis*.

Effect of the presence of *R. insecticola* on aphids surviving parasitoid attack

Aphids from the surviving Linyi-1 and Linyi-2 clones and control aphids after *A. gifuensis* attack showed no difference in fecundity ($F_{2, 144} = 1.03$, $P = 0.36$; Fig. 4A), but a slight but significant 1.1-fold increase in the survival rate ($\chi^2 = 10.67$, $df = 2$, $P = 0.005$; Fig. 4B) compared to Linyi-NA. However, the surviving individuals of the Linyi-1 and Linyi-2 clones, and the control after *A. asychis* attack differed in their fecundity ($F_{2, 125} = 162.50$, $P < 0.001$) and survival rate ($\chi^2 = 84.44$, $df = 2$, $P < 0.001$). The Linyi-1 and Linyi-2 clones showed a strong decrease in both fecundity (1.70- and 1.56- fold, respectively; Fig. 5A) and survival rate (1.61- and 1.57- fold, respectively; Fig. 5B) compared to the Linyi-NA clone.

Discussion

Symbiont-mediated protection has been reported in a wide variety of insects. Pea aphids harboring the facultative endosymbiont *R. insecticola* reduced susceptibility to a fungal pathogen infection and affected a dietary breadth of the host aphids (Scarborough et al. 2005; Ferrari et al. 2007). More recently, an isolate of this symbiont was also involved in the aphid defense against parasitoid wasps (Vorburger et al. 2010). Since the aphid *S. avenae* is the most devastating insect for winter wheat in China (Xu et al. 2011) and *R. insecticola* is widely present in *S. avenae* individuals collected from the fields of this country (Li et al. 2014; Luo et al. 2016), we therefore investigated whether the presence of this symbiont could confer a benefit to the aphids against parasitoids used as biological control agents.

In a previous study, we observed that transferring *R. insecticola* strains to the *S. avenae* Neixiang genotype significantly increased the rate of parasitism by *A. asychis* (from 12 to 58%), suggesting an increased susceptibility of aphids (Luo et al. 2017b). However, the presence of *R. insecticola* reduced the parasitoid emergence rate from 90% to 70%, due to an increase in wasp larval mortality and a slightly reduced weight (~5%) of emerging parasitoids. Since the overall net production of parasitoid offspring was about 4-fold higher in the presence of *R. insecticola*, the presence of the facultative symbiont appeared to be advantageous for the parasitoid instead of protecting the aphid. Here, using a different genotype of *S. avenae* from Linyi, we observed a similar trend for parasitism by *A. asychis*: the presence of *R. insecticola* increased the parasitism rate (from 38 to 62%) but the development of the parasitoid was strongly affected with a decrease in emergence rate from 86% to 51% and weight of the emerged wasps (~9% lower). Similarly, emerging evidences suggested that the present of symbionts could also increase infection/predation risks of virus/predation to the hosts, respectively (Dodson et al. 2014; Ramírez-Cáceres et al. 2019).

Moreover, the overall net production of parasitoid offspring was the same between the two different Linyi lines. Thus, the presence of *R. insecticola* is not only detrimental to the aphid (more are parasitized and die mummified), but also for the parasitoid wasp whose success and weight of the offspring are reduced. In this case, parasitizing the infected aphids is therefore counterproductive for *A. asychis* since it needs to invest more energy to achieve parasitism and obtain the same final production of offspring. Most of the effects observed seemed specific to *A.*

asychis since the presence of *R. insecticola* had no significant effect on the rate of parasitism of aphids or of emergence rate with *A. gifuensis* (Ye et al. 2018). The only differences were in the weight of emerged parasitoids, which was more affected for *A. gifuensis* wasp than for *A. asychis*. The differences on resisting to parasitoids may also explain the large multilocus variations within a *S. avenae* population in China (Xin et al. 2014), along with this aphid species is a major agricultural pest of winter wheat, suggesting more efforts are needed in *S. avenae* control than other aphid species in China. For example, over two genotypes of *S. avenae* population of a region used in the experiment seem more reasonable, providing thereby more reliable knowledge in *S. avenae* control.

In particular, the present study has shown increased susceptibility for *A. asychis* of *R. insecticola*-infected aphids, an increase that could have different causes. Plants attacked by herbivores, including aphids, release specific volatile blends that attract their natural enemies (Pare and Tumlinson 1999; Bradburne and Mithen 2000; Sasso et al. 2007; Stahl et al. 2017; Turlings and Erb 2018). To date, there are only few studies on the effect of aphid facultative symbionts on the attractiveness of aphid-infested plants to parasitoids. Frago et al. (2017) observed that plants on which *H. defensa*-bearing aphids fed were significantly less attractive to *A. ervi* wasps, which therefore reduced the recruitment of parasitic wasps and increased aphid fitness. This difference was related to qualitative and quantitative changes in the volatile substances emitted by the attacked plant. It is therefore possible that the presence of *R. insecticola* changed the volatile emissions of the plant or volatile blends making it more susceptible to *A. asychis* or increasing its foraging behavior. *R. insecticola*-infected aphids may also secrete less β -farnesene alarm hormone (Bowers et al. 1972) leading to less protection of the aphid population. Differences in the amount of β -farnesene produced between *H. defensa*-infected and uninfected aphids have been observed (Oliver et al. 2012). Whether it could be the same for Linyi lines remains to be further investigated, although Ramírez-Cáceres et al. (2019) showed that β -farnesene had a bit lower, but no significant different, concentration on Chilean clones of *S. avenae* infected with *R. insecticola* feeding on the wheat. Additionally, some studies have unraveled that symbiont *H. defensa* could affect (positive or negative) host aphid's body size (Castañeda et al. 2010; Vorburger and Gousskov 2011), along with we did not quantify aphid's body size in the current study. Thus, it is not possible to exclude that the increased susceptibility may be induced by aphid body size.

The body weight of emerged *A. gifuensis* and *A. asychis* wasps was significantly reduced in the presence of *R. insecticola*, indicating that the symbiont negatively affects the growth of the parasitoid larvae. Ye et al. (2018) also suggested that the symbiont could decrease parasitoid survival in natural *S. avenae* populations. The possible reason of adverse impacts on developing parasitoid larva may due to the production of toxin(s) or the competition on the use of some metabolites. The presence of toxin(s) or nutritional competition may also explain the higher mortality rate specific to the *A. asychis* species during development. The facultative bacterial symbionts of aphids are known to secrete toxin(s) that kill developing wasp larvae (Degnan et al. 2009; Hansen et al. 2011; Brandt et al. 2017; Oliver and Higashi 2019) and competition for metabolites between symbionts and parasitoid larvae has been described in *Drosophila* (Paredes et al. 2016). For both species, fitness of newly emerged parasitoids may have decreased since the size and fitness of female parasitoids are positively correlated (West et al. 1996; Cloutier et al. 2000; Beukeboom 2018).

Another possible explanation for the higher mortality of *A. asychis* could be related to its behavior. *A. asychis* females induce a paralysis of the host and feed on it before laying. Although aphids can recover from paralysis,

they could be strongly affected by wasp feeding (Cate et al. 1974; Chorney 1979; Bai and Mackauer 1990). If one female does not use the same aphid to feed and lay, another might accept this host because of competition, and its offspring and the host may not survive due to the lower quality of the host. Moreover, although no information is yet available, the injection of venom of *A. asychis* during oviposition could be more harmful for infected aphids (and more harmful than that of *A. gifuensis*), and therefore explain the increased mortality of parasitized aphids.

Under normal conditions, the presence of *R. insecticola* in the Linyi genotype increases the longevity of the aphid by about 5 days, but no net reproductive gain was observed due to a decrease in the birth rate (Luo et al. 2017a). After the attack of *A. gifuensis*, the survival aphids of the three Linyi lines showed a similar longevity and fecundity, close to those of the not attacked normal Linyi-NA. This suggests that stress has suppressed the survival advantage of aphids conferred by *R. insecticola*. This effect of stress is much more visible with *A. asychis*. The presence of *R. insecticola* strongly reduced the aphid survival (about 6 days compared to uninfected condition and up to 10 days compared to unstressed conditions) and reduced accordingly their fecundity compared to the Linyi-NA line. Because we do not know if the surviving aphids were simply stressed by the presence of the parasitoids or pricked (and venom injection) without oviposition or submitted to a complete sequence of oviposition followed by the failure of the egg to develop, the reason for this difference is difficult to explain. However, we can conclude that the two parasitoid species induced a strong fitness cost on the aphids having survived the attack, which depends upon the presence of the facultative symbiont, the effect being more pronounced for *A. asychis*.

Studies attempting to measure the cost of carrying facultative symbionts in aphids have yielded a wide range of results (Feldhaar 2011; Cayetano et al. 2015; Hrcek et al. 2016; Martinez et al. 2018): Some have reported that symbionts can be beneficial and lead to an increase in the fecundity of the host, while others have shown that a cost, such as a reduction in aphid longevity, may exist in the symbiotic relationship. For example, while adults from *Aphis fabae* clones harboring *H. defensa* had significantly higher body mass and offspring production (Castañeda et al. 2010), strongly protective isolates of *H. defensa* reduced lifespan and lifetime reproduction more than weakly protective isolates, suggesting that more protective symbionts are more costly to the host (Cayetano et al. 2015). *R. insecticola* showed either a positive, negative or neutral effect on the fecundity of *A. pisum* on clover depending on the aphid genotype, the *R. insecticola* isolates and the clover species (Tsuchida et al. 2004; Ferrari et al. 2007; Leonardo 2004; McLean et al. 2011). Interestingly, the *R. insecticola* strain increasing the fecundity of the pea aphid on clover also increased the survival and reproduction of the non-host aphid *Megoura crassicauda* in the same host plant (Tsuchida et al. 2010). Thus, this specific *R. insecticola* strain can increase the performance of two distinct aphid species on a specific host-plant environment. The mechanism(s) involved are still unknown.

The findings of this study thus agree with and corroborate previous studies showing that the cost/benefit of the presence of a facultative symbiont is dependent on the context and genotypes. It will be interesting in the future to introduce the *R. insecticola* isolates used here in different species of aphids to analyze the phenotypic effects and to sequence their genomes to compare them with those of already characterized strains. Moreover, the work on resisting to fungal pathogens is necessary, a main role of the symbiont *R. insecticola*. Finally, it will be indispensable to extend our study to more natural conditions to conclude whether the presence of *R. insecticola* will be beneficial for the aphid host or the parasitoid wasps used as biological agents under field conditions (Ye et al. 2018).

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Author contributions

ZQH, HYZ and CL perceived the idea and designed the study; CL and ZQH collected the data; CL, LSM, and ND performed data analyses; CL, JLG, LSM, ZQH, ND and MP wrote the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

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Figures

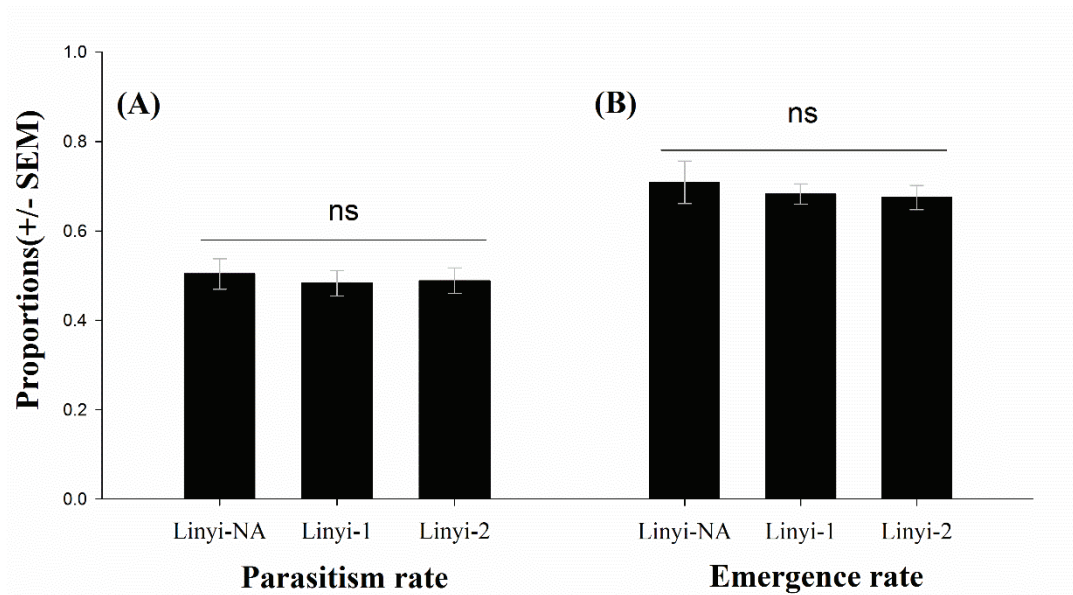


Figure 1. Effect of *R. insecticola* infection on the parasitism rate (A) of *S. avenae* aphids by *A. gifuensis* and the emergence rate (B). Linyi-NA is devoid of facultative symbionts, and Linyi-1 and Linyi-2 host different *R. insecticola* strains. Each value is the mean \pm SEM. 'ns' above bars indicates no significant difference ($P > 0.05$).

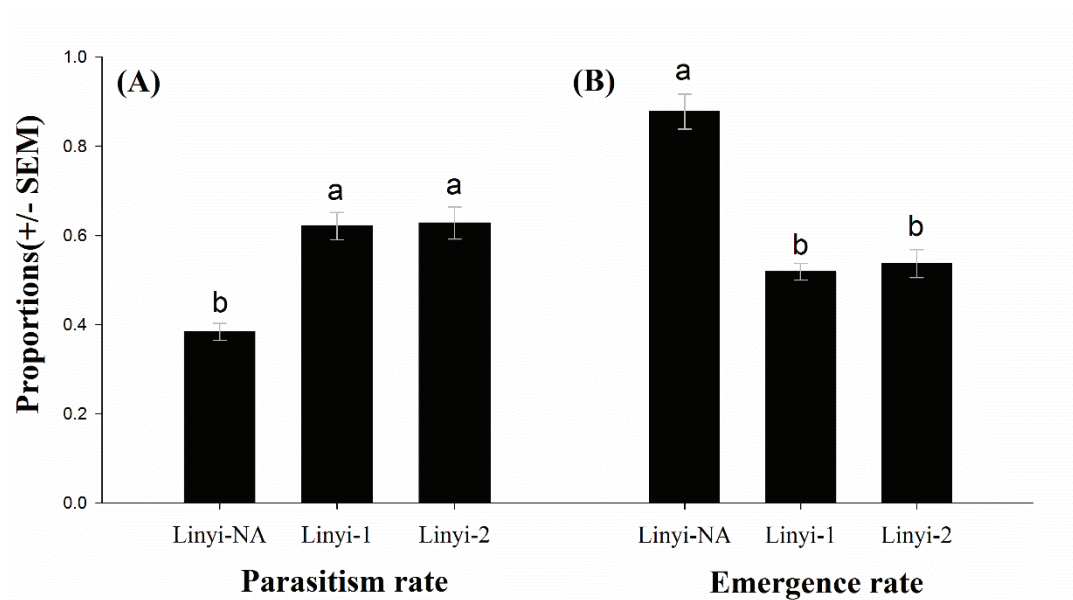


Figure 2. Effect of *R. insecticola* infection on the parasitism rate (A) of *S. avenae* aphids by *A. asychis* and the emergence rate (B). Linyi-NA is free of facultative symbionts, and Linyi-1 and Linyi-2 host different *R. insecticola* strains. Each value is the mean \pm SEM. Different letters above bars within each panel indicate significant differences ($P < 0.05$).

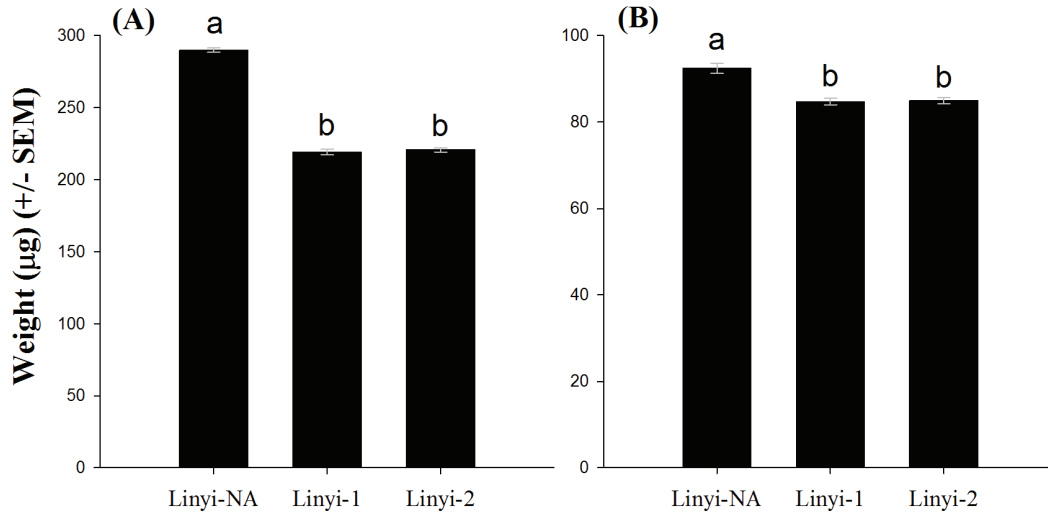


Figure 3. Effect of *R. insecticola* on the weight of emerged wasps [*A. gifuensis* (A) and *A. asychis* (B)]. Linyi-NA is devoid of facultative symbionts (only *Buchnera*); Linyi-1 and Linyi-2 harbor different *R. insecticola* strains. Each value is the mean of emerged wasp weights \pm SEM. Different letters above bars indicate significant differences ($P < 0.05$).

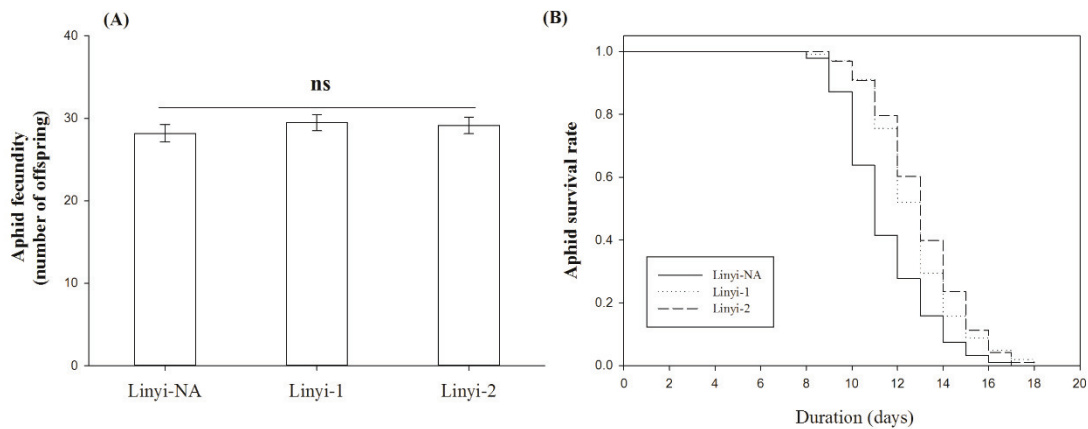


Figure 4. Effect of the symbiotic status on the fecundity and survival rate of unparasitized aphid lines under the stress of *A. gifuensis* wasp attack. (A): number of offspring per aphid in the three aphid lines. 'ns' above bars indicates no significant difference ($P > 0.05$); (B): survival rate of the three aphid lines. All results are presented as mean values \pm SEM. The three different symbiotic statuses are presented: Linyi-NA is free of facultative symbionts. Linyi-1 and Linyi-2 harbor different *R. insecticola* strains.

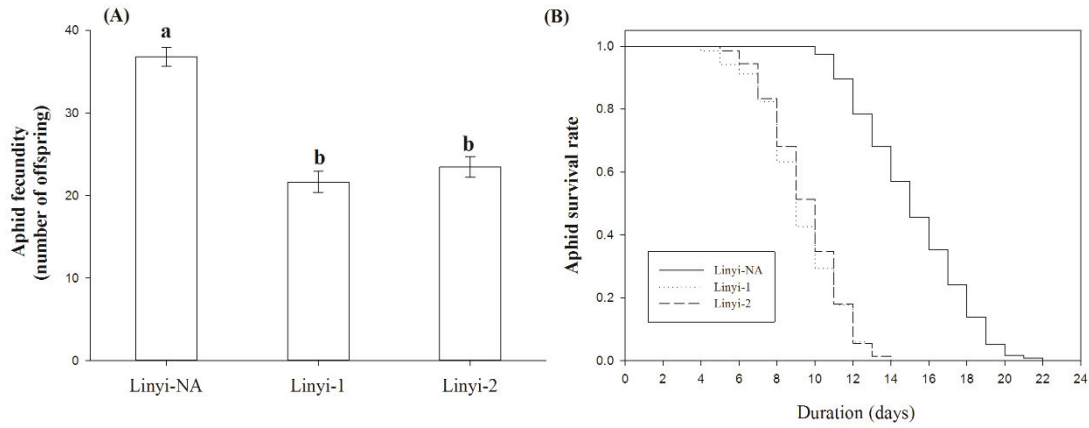


Figure 5. Effect of the symbiotic status on the fecundity and survival rate of unparasitized aphid lines under the stress of *A. asychis* wasp attack. (A): number of offspring generated per aphid in the three aphid lines. ‘ns’ above bars indicates no significant difference ($P > 0.05$); (B): survival rate in the three aphid lines. All results are presented as mean values \pm SEM and different letters (a, b) show significant differences. Different symbiotic statuses are presented: Linyi-NA is devoid of facultative symbionts (only *Buchnera*); Linyi-1 and Linyi-2 harbor different *R. insecticola* strains.

Supplementary tables

Table S1. Collection information and identification of secondary symbionts in different isofemale strains of *S. avenae*.

Collection location.	Longitude/latitude	<i>Buchnera</i>	<i>R. insecticola</i>	<i>H. defensa</i>	<i>S. symbiotica</i>	<i>Spiroplasma</i>	<i>Arsenophonus</i>	<i>Rickettsia</i>	<i>Rickettsiella</i>	PAXS
Linyi-NA, Shandong	35.05°N, 118.35°E	+	-	-	-	-	-	-	-	-
Yangpingguan-1, Shaanxi	34.8°N, 105.6°E	+	+	-	-	-	-	-	-	-
Yangpingguan-2, Shaanxi	34.8°N, 105.6°E	+	+	-	-	-	-	-	-	-

+ indicates that the symbiont was present in the isofemale strains examined.

Table S2. Aphid clonal lines used in this study.

Aphid lines	Facultative symbiont	Year introduced to lab
Linyi-NA	Uninfected	2014
Linyi-1	<i>R. insecticola</i> - YPG1	2014
Linyi-2	<i>R. insecticola</i> - YPG2	2014

Conclusion and perspectives publication 1

Conclusion

Various studies have examined the effect of secondary symbionts in *S. avenae*. *Hamiltonella defensa* and *Regiella insecticola* are among the major facultative symbionts in aphid species and are present in the natural clones of *S. avenae* (Oliver et al. 2010; Łukasik et al. 2013b; Wang et al. 2016; Luo et al. 2016). Both symbionts offer a wide variety of protection services to their hosts against their natural enemies including parasitic wasps, predators and fungal pathogens (Oliver et al. 2003; Łukasik et al. 2013a).

The impact of natural *H. defensa* infections on life-history traits of *S. avenae* was previously explored on three plants (i.e. wheat, oat, and rye) (Li et al. 2018). Compared to the same antibiotic cured lines, *H. defensa*-infected lines were less fertile on wheat and oat, but not on rye, suggesting cost of infection for the aphid on susceptible host plants.

In their study the authors also showed that parasitism rates by *Aphidius gifuensis* did not differ from between *H. defensa*-infected and cured lines. They proposed that this lack of protection could be related to the type of APSE infecting *H. defensa* in their aphid clones: indeed, all clones used had *H. defensa* infected with APSE-2 that confers partial or insufficient protection to *A. ervi* in pea aphids (Oliver and Higashi 2019). These results were consistent with those of Łukasik et al. (2013b) also showing that the infection with *H. defensa* did not reduce the susceptibility of *S. avenae* to *A. ervi* and *Ephedrus plagiator*. Furthermore, symbiont infection had no effect on parasitoid development time or adult size. However, in experiments of choice, it was found that experienced parasitoid females preferred to oviposit in uninfected hosts than infected ones, showing the impacts of *H. defensa* on parasitoid searching behavior (Łukasik et al., 2013b). The reasons why *H. defensa* is maintained in *S. avenae* in the fields also require further studies.

Wang et al. (2016) also showed that infection of *S. avenae* with *R. insecticola* did not have significant advantages for the fitness of its aphid host on wheat and oat in terms of developmental time and fecundity, and that it even slightly reduced the fitness of *S. avenae* on rye. Such results indicate that this secondary endosymbiont could have little or no effect in facilitating the use of particular plants for its aphid host. We have demonstrated that *R. insecticola* can protect *S. avenae* from some but not all parasitoid species similarly to the previous results of von Burg et al. (2008) that some of these bacterial strains provide a protective phenotype. This broadens the panel of phenotypic effects conferred by *R. insecticola*

and confirms that the cost/benefit ratio of the presence of a facultative symbiont depends on the context and genotypes. Such finding is also important in an applied context since as it may compromise the effectiveness of biological control with parasitoids.

Perspectives

Recent molecular analysis has characterized all bacterial endosymbionts in ten genetically defined *S. avenae* clones from Germany (Alkhedir et al. 2015). Phylogenetic analysis showed that *Buchnera* from *Sitobion* was related to that in Macrosiphoni. The analysis of the secondary endosymbionts indicated no host relationship between *H. defensa* and *R. insecticola* from *Sitobion* and those from other aphid species. This highlights that aphid symbionts evolve with their host and that many strains conferring different phenotypes may be present in nature (Vorburger and Perlman 2018). The fact that the only protective strain genotype to date (Hansen et al. 2011) possesses eukaryotic toxins and mechanism necessary to externalize them, absent from the non-protective strain, encourages us to further analyze the genome of the *R. insecticola* strains used in our study. This could help in the future to provide clues to decipher the molecular basis of this conferred resistance. It will be also of interest to transfer this strain of *Regiella* to pea aphid and other aphid species to test the protection conferred against a larger panel of parasitoids.

At least in the context of biological control, it is important to check whether, under parasitoid pressure, the protective bacteria can spread through *S. avenae* populations, therefore increasing its resistance.

Classifying parasitoids according to their specialization degree

The life history traits of parasitoids are highly variable at both interspecific and intraspecific scales and a considerable number of studies have been conducted on their evolution (For reviews, Jervis et al. 2008; Godfray 2016; Mayhew 2016). Due to the lifestyle of a parasitoid, the allocation of resources to different traits depends on the quality of the host that is influenced by factors such as host species, developmental stage, body size, defense capacities before (behavioral) and after (physiological) parasitism, nutritional status and parasitic state. Situations of conspecific superparasitism (multiple oviposition by parasitoids of the same species) or multiparasitism (oviposition by parasitoids of different species) can also reduce the parasitoid fitness. In solitary species, in case of superparasitism or multiparasitism, a single parasitoid can complete its larval development, the other individuals being eliminated (Godfray 1994). Host-parasitoid interactions therefore represent a unique case of dependence between the fitness of an organism and the exploitation of a food resource. The state of development of the exploited host is also an important factor for the fitness of parasitoids due to differences in the amount of available food resources and in the defense capacities of each developmental stage. The host is in turn strongly selected to prevent the success of parasitism (Kraaijeveld and Godfray 1997). The hosts have therefore evolved a wide array of mechanisms to avoid parasitism, including the expression of behavioral, morphological, and immunological defenses. Moreover, as we have seen in the previous chapters, the presence of symbionts in the host may impact the success of the parasitoid and/or the fitness of its offspring. Parasitoids have a varying degree of specialization, ranging from "oligophagous" or "specialist" species that exploit a limited number of host species to "polyphagous" species that are more "generalist" with a wider host spectrum (Strand and Obrycki 1996; Loxdale et al. 2011). In parasitoids exploiting several host species, populations may diverge and adapt to available hosts in their areas.

In the parasitoid-host relationship, the "optimal foraging strategy" was regarded as a central paradigm of the host selection model that is determined by the correlation between the host choice of the parasitoid mother and the survival and development of the resulting progeny (for review Pyke et al. 1977; Desneux et al. 2009 a, b). The first two papers on the theory of optimal foraging were published back-to-back in 1966. The paper by MacArthur and Pianka (1966) discusses two problems, (a) what type of prey an effective predator should include in its diet, and (b) what types of food patches should be included in a predator's route. Optimal strategies for both problems are found by minimizing the time spent searching for and pursuing prey for the prey choice problem or the time spent traveling between patches and hunting in each patch

for the patch choice problem. Emlen's (1966) paper is much more mathematical (1966) and it considers a predator to decide whether or not to accept food items based on their value and the time required to handle them. One implication of Emlen's (1966) model is that when both types of prey are rare, the predator should not be selective. If prey is relatively common, predators should be more selective, but the pattern of selectivity depends on the relative quality of the prey and not just on its quality. Since then, this theory has sparked numerous studies and intellectual debates (for review Schoene 1987).

This optimal theory of foraging has generally been considered in the context of the preference-performance hypothesis (PPH) (Jaenike 1978; Gripenberg et al. 2010): This relationship predicts a positive correlation between (i) the choice of the host plant by adult females during oviposition (preference) and (ii) the successful development of their offspring feeding on their plants (performance). The PPH states that females will preferentially oviposit on hosts that may maximize the survival and performance of their larvae. This prediction of the maximization of fitness through survival of the offspring is also known as the "naïve adaptation" or the "mother knows best" hypothesis. This relationship has been demonstrated in a large number of herbivorous organisms (Fig. 11) (Jaenike 1978; Thompson 1988; Nylin and Janz 1993; Craig 2004; Gripenberg et al. 2010), but also in predators (Sadeghi and Gilbert 1999) and some parasitoids (Driessen et al. 1991; Brodeur et al. 1998; Desneux et al. 2009 a, b).

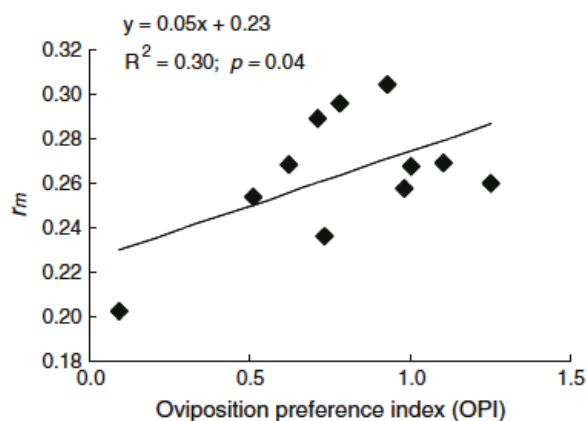


Fig. 11. Relationship between adult oviposition preference and intrinsic rate of increase (r_m) of *P. xylostella* among 11 wild crucifers. The OPI indicates the relative oviposition preference for a given crucifer over Chinese cabbage, and therefore a higher OPI indicates a higher oviposition preference of *P. xylostella* for a given crucifer (from Zhang et al. 2012)

In the parasitoids, the preference is for the behavioral host range (number of host species they are able to attack) and the performance is for the physiological host range (number of host species in which they are able to complete their life cycle). This relationship has been

demonstrated in particular in specialist organisms for obvious reasons (Driessen et al. 1991, Brodeur et al. 1998, Desneux et al. 2009a, Gripenberg et al. 2010): specialist parasitoids use chemical and physical signals related to the hosts they have specialized on. However, this relationship should not exist for generalists since they use general physical and chemical signals and attack a large number of hosts more or less adapted to the development of their larvae. However, very few studies have tested this relationship in generalist parasitoids, and it is important to note that these studies have tested the existence of this relationship on a small number of host species (maximum 3) and considered, in addition, hosts belonging to the same genus or tribe (Li et al. 2009; Kos et al. 2012).

This is the context in which we tested the existence of the preference-performance relationship in three parasitoid species considered as generalists. This work is described in Publication 2.

Publication 2. The preference-performance relationship as a means of classifying parasitoids according to their specialization degree

The preference and performance of specialized parasitoids should be correlated contrary to generalist parasitoids. Many studies have shown supports for the PPH hypothesis for specialized parasitoids species (Driessen et al. 1991; Brodeur et al. 1998). However, such a relationship may not be common in the case of generalist parasitoids (Gripenberg et al. 2010; Chesnais et al. 2015), drawbacks being that these studies tested only a few host species (a maximum of 3) and host species belonging to the same tribe or genus [possibly inducing bias in assessing the preference-performance correlation (Poulin and Mouillot 2005)].

The PPH relationship was analyzed for three species of aphid parasitoids currently considered as generalist species: *Aphelinus abdominalis* (Aphelinidae), *Aphidius ervi* (Braconidae), and *Diaeretiella rapae* (Braconidae) using 12 species of aphids from 2 different tribes (Aphidini and Macrosiphini). In our laboratory conditions, each aphid species was maintained on 6 different host plants. Additionally, since secondary endosymbionts could modulate the preference and/or performance of parasitoids (Monticelli et al. 2019), we further screened for the presence of nine secondary endosymbionts in all aphid colonies used.

Publication 2


The preference-performance relationship as a means of classifying parasitoids according to their specialization degree

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The preference–performance relationship as a means of classifying parasitoids according to their specialization degree

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Abstract

Host range in parasitoids could be described by the preference–performance hypothesis (PPH) where preference is defined as host acceptance and performance is defined as the sum of all species on which parasitoid offspring can complete their life cycle. The PPH predicts that highly suitable hosts will be preferred by ovipositing females. However, generalist parasitoids may not conform to this hypothesis if they attack a large range of hosts of varying suitability. Under laboratory conditions, we tested the PPH relationship of three aphid parasitoids currently considered as generalist species (*Aphelinus abdominalis*, *Aphidius ervi*, *Diaeretiella rapae*). As expected, the three parasitoids species showed low selectivity, i.e., females stung all aphid species encountered (at least in some extent). However, depending on the parasitoid species, only 42%–58% of aphid species enabled producing parasitoid offspring. We did not find a correlation between the extent of preference and the performance of three generalist aphid parasitoids. For *A. ervi*, host phylogeny is also important as females showed higher attack and developmental rates on hosts closely related to the most suitable one. In addition, traits such as (a) the presence of protective secondary endosymbionts, for example, *Hamiltonella defensa* detected in *Aphis fabae* and *Metopolophium dirhodum* and (b) the sequestration of plant toxins as defense mechanism against parasitism, for example, in *Aphis nerii* and *Brevicoryne brassicae*, were likely at play to some extent in narrowing parasitoid host range. The lack of PPH relationship involved a low selectivity leading to a high adaptability, as well as selection pressure; the combination of which enabled the production of offspring in a new host species or a new environment. Testing for PPH relationships in parasitoids may provide useful cues to classify parasitoids in terms of specialization degree.

KEY WORDS

aphid, generalist parasitoids, host range, preference–performance hypothesis, specialization

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1 | INTRODUCTION

Host specificity, and more broadly diet breadth, has been described by the preference–performance hypothesis (PPH) of Jaenike (1978). It predicts a positive relationship between the choice of adult females (preference) and the degree of successful offspring development (performance). Various studies have shown support for the preference–performance hypothesis for specialized phytophagous arthropods (Craig, Itami, & Price, 1989; Gripenberg, Mayhew, Parnell, & Roslin, 2010; Jaenike, 1978; Nylin & Janz, 1993; Thompson, 1988) as well as for specialized predators (Sadeghi & Gilbert, 1999) and parasitoids (Brodeur, Geervliet, & Vet, 1998; Desneux, Barta, Hoelmer, Hopper, & Heimpel, 2009; Driessen et al., 1991). However, it has been argued that such relationships may not be common in the case of generalist arthropods (Chesnais, Ameline, Doury, Roux, & Couty, 2015; Eben, Benrey, Sivinski, & Aluja, 2000; Gripenberg et al., 2010).

Parasitoids are insects of which free-living adult females deposit eggs in, on, or near hosts and immature stages develop by host consumption (parasitic stage; Godfray, 1994). Hence, the host represents the only food source for the parasitoid larvae and female choice to lay its egg is decisive. The selection of hosts by parasitoids involves the detection of physical and/or chemical cues from the other trophic levels (e.g., host species and/or host plants) (Mackauer, Michaud, & Völkl, 1996; Vet & Dicke, 1992; Vinson, 1985), and the host specificity of parasitoids may be mainly shaped by infochemicals, i.e., chemicals emitted by host and/or host plant (Afsheen, Wang, Li, Zhu, & Lou, 2008). Specialist parasitoids may use more specific cues related to their hosts (Barbosa, 1988; McCormick, Unsicker, & Gershenson, 2012; Vet & Dicke, 1992). This is the case, for example, for *Microplitis croceipes* that uses host kairomones from a variety of host-related sources (e.g., frass, hemolymph, and salivary secretions; Alborn, Lewis, & Tumlinson, 1995; Jones, Lewis, Bowman, Beroza, & Bierl, 1971). By contrast, generalist parasitoids often use more generalized cues to identify potential host species (Vet & Dicke, 1992). For example, the generalist fly parasitoid *Aphaereta minuta* does not use host-derived chemical cues to select host larvae and attacks almost all hosts that are present in encountered decaying materials (Vet, 1985).

Assessing PPH relationships in parasitoids may provide useful clues to classify parasitoids in terms of specialization degree; i.e., specialized parasitoids may show significant PPH relationships, whereas generalist ones rarely do. However, several studies have reported positive preference–performance relationships in parasitoids considered to be generalists (Kos et al., 2012; Li, Miller, & Sun, 2009). It should be noted though that all of these studies (a) tested only a few host species (a maximum of 3), and (b) the host species belonged to the same tribes or genus thus representing a possible bias in assessing the preference–performance correlation (Poulin & Mouillot in, 2005). Such reported positive PPH relationships may actually be false positives in the sense that these studies were not designed per se to assess the link between the preference of females and the performance of offspring in the context of the PPH (Gripenberg et al., 2010).

In this context, we assessed the PPH relationship in three aphid parasitoid species *Aphelinus abdominalis* (Aphelinidae), *Aphidius ervi* (Braconidae), and *Diaeretiella rapae* (Braconidae) that have been considered generalists, i.e., attacking a broad phylogenetic range of aphids (Honek, Jarosik, Lapchin, & Rabasse, 1998; Kavallieratos et al., 2004), through characterization of the behavioral (preference) and physiological (performance) determinants of host specificity of these parasitoid species under laboratory conditions. To achieve this, we used twelve aphid species that feed on six different host plants and spread over two different tribes (Aphidini and Macrosiphini) within the subfamily Aphidinae. They were chosen to cover a broad phylogenetic range of aphid species (Coeur d'Acier, Joussetin, Martin, & Rasplus, 2007; Desneux, Barta, Hoelmer, et al., 2009; von Dohlen, Rowe, & Heie, 2006). In addition, we calculated the host specificity index S_{TD}^* from Poulin and Mouillot (2005) to classify the parasitoids according to their host specificity; doing so we identified an endpoint for quantifying where these species lie on a generalist–specialist continuum. For this, we considered also previous results on specialist parasitoids generated by our laboratory (Desneux, Barta, Hoelmer, et al., 2009; Monticelli, 2018). Finally, because various ecological factors such as secondary endosymbionts (Hopkinson, Zalucki, & Murray, 2013; Oliver, Russell, Moran, & Hunter, 2003) may modulate the preference and/or performance of parasitoids (Monticelli, Outreman, Frago, & Desneux, 2019), each aphid colony was screened for the presence of nine secondary endosymbionts.

2 | MATERIALS AND METHODS

2.1 | Biological materials

All the aphid colonies used in the study were initiated from individuals collected in France, and all colonies were mixtures of clones. The description of the aphid species, their color, hosts plants, the aphid tribe, and the number of replications performed in experiments for each parasitoid species are reported in Table 1.

Aphelinus abdominalis and *A. ervi* colonies were initiated from parasitoids naturally colonizing *Macrosiphum euphorbiae* on *S. lycopersicum* in the INRA Sophia Antipolis greenhouse complex (57 [21 males and 36 females] and 61 individuals [19 males and 42 females], respectively). *Diaeretiella rapae* colony was initiated from parasitoids naturally colonizing *Brevicoryne brassicae* in Brittany (29 [12 males and 17 females]). They were reared for 4–5 generations in the laboratory before starting the experiments. All parasitoids were maintained on their principal hosts (most frequent and/or suitable host): *Acyrtosiphon pisum* for *A. abdominalis* (Hullé, Turpeau, & Chaubet, 2006; Pons, Lumbierres, Antoni, & Sary, 2011) and *A. ervi* (Kavallieratos et al., 2004; Nguyen et al., 2018) wasps and *B. brassicae* for *D. rapae* (Desneux, Rabasse, Ballanger, & Kaiser, 2006; Kavallieratos et al., 2004) in climatic cabinets (23 ± 2°C, RH 65 ± 5% and photoperiod 16:8 L:D hr). Before experiments, the parasitized aphids were retrieved at the mummy stage and isolated in plastic Petri dishes. After adult emergence, females were mated and

TABLE 1 Aphid tribe, species, their color, host plants, and the number of replicates for *Aphelinus abdominalis*, *Aphidius ervi*, and *Diaeretiella rapae*, respectively

Tribe	Species	Aphid color	Host plant species	Replication		
				Preference	Performance	Endosymbiont presence
Aphidini	<i>Aphis fabae</i>	Black	Bean (<i>Vicia fabae</i>)	(32, 52, 30)	(n/a, 64, n/a)	(6)
	<i>Aphis gossypii</i>	Yellow	Squash (<i>Cucurbita moschata</i>)	(32, 47, 30)	(n/a, 69, 59)	(5)
	<i>Aphis craccivora</i>	Black	Bean (<i>Vicia fabae</i>)	(31, 70, 31)	(41, 45, n/a)	(5)
	<i>Aphis nerii</i>	Yellow	Milkweed (<i>Asclepias</i> sp.)	(37, 63, 31)	(58, 46, 70)	(5)
	<i>Rhopalosiphum padi</i>	Black	Wheat (<i>Hordeum vulgare</i>)	(33, 67, 32)	(66, 59, 73)	(4)
	<i>Schizaphis graminum</i>	Green	Wheat (<i>Hordeum vulgare</i>)	(32, 58, 40)	(63, 53, 68)	(5)
	Macrosiphini	<i>Brevicoryne brassicae</i>	Green	Cabbage (<i>Brassica oleracea</i>)	(33, 109, 46)	(65, 56, 76)
<i>Myzus persicae</i>		Green or Red	Cabbage (<i>Brassica oleracea</i>)	(66, 127, 80)	(181, 99, 171)	(10)
<i>Sitobion avenae</i>		Green	Wheat (<i>Hordeum vulgare</i>)	(37, 59, 45)	(82, 54, 78)	(5)
<i>Metopolophium dirhodum</i>		Yellow	Wheat (<i>Hordeum vulgare</i>)	(32, 55, 30)	(80, 52, n/a)	(6)
<i>Macrosiphum euphorbiae</i>		Green	Potato (<i>Solanum tuberosum</i>) Tomato (<i>Solanum lycopersicum</i>)	(64, 107, 60)	(221, 138, 118)	(10)
<i>Acyrthosiphon pisum</i>		Green	Bean (<i>Vicia fabae</i>)	(32, 41, 30)	(80, 42, 61)	(6)

Note: All aphid species tested belonged to the family Aphididae (subfamily Aphidinae), Blackman and Eastop (2006). All aphid colonies were initiated from ≥ 200 collected individuals naturally colonizing fields in France during 2013–2014 and infesting their respective host species. Two strains of *M. euphorbiae* were maintained on their respective collection plant species: *S. tuberosum* and *S. lycopersicum* (indicated as P and T for potato and tomato in the text, respectively). Two strains of *M. persicae* were used (one green and one red). All aphids were maintained (for 35–45 generations) on their host plant in a ventilated cage (60 × 60 × 60 cm) covered by mesh, under controlled conditions (23 ± 2°C, RH 65 ± 5% and photoperiod 16:8 hr L:D). n/a means that data are nonavailable due to the unsuccessful sting of the parasitoids in these aphid species.

fed a honey solution (50% water + 50% honey) for at least 24 hr. Parasitoids used for all experiments were 24–48 hr old, used only once, and had never been in contact with plants or aphids (i.e., no experience before being tested).

2.2 | Experiment 1: Parasitoid preference and performance measurements

Parasitoid preference. Preference for the different host species for all three parasitoid species was estimated by observing parasitoid behavior when they encountered individuals of the different aphid species tested. Three parasitoid behavioral steps were identified: detection, acceptance, and oviposition (see Desneux, Barta, Hoelmer, et al., 2009). For *A. ervi* and *D. rapae*, detection was defined as physical contact between aphid and parasitoid, followed by antennal palpation. Acceptance was described as the parasitoid abdomen bending underneath its thorax in the direction of the aphid, and sting was described as the introduction of the ovipositor into the aphid. In case of *A. abdominalis* wasps, effective detection was described as right-left bounces behind the aphid followed by parasitoid acceptance, i.e., 180°

rotation of the female, and the start of ovipositor use. A sting was described as an ovipositor introduction into aphids lasting at least 20 s and not ending as a result of aphid defensive behaviors (Wahab, 1985). Aphid defensive behaviors were also recorded, and three behaviors were considered defensive: kicking, cornicle secretion, and escape. For the analyses, all defensive behaviors were grouped.

For each replicate, one leaf of one host plant was placed upside down under a binocular magnifier (8×). One individual from one aphid species was placed on the leaf with a fine brush. After 5 min of establishment, one mated female parasitoid was introduced. When the parasitoid touched the leaf, the observation began and the parasitoid's behavior was noted over 5 min for *A. ervi* and *D. rapae* (short stinging time) and over 10 min for *A. abdominalis* (long stinging time, Wahab, 1985). The preference experiment was stopped after the 5 or 10 min or when the parasitoid exhibited oviposition behavior. Each parasitoid and aphid species was tested randomly every experimental day.

Aphid size is known to have an impact on the parasitoid host selection process (Wyckhuys et al., 2008). Hence, the aphids used in the experiment were all of the equivalent size of 3rd- and 2nd-instar *A. pisum* for *A. abdominalis* and *A. ervi* wasps, respectively, and

equivalent to 3rd-instar *B. brassicae* for *D. rapae*, i.e., the known instar preferred by these parasitoids for oviposition (Henry, Gillespie, & Roitberg, 2005; Khakasa, Mohamed, Lagat, Khamis, & Tanga, 2016; Wahab, 1985).

Parasitoid performance. The physiological host range was established by monitoring parasitoid development in the different aphid species. Aphids stung in part 1 of the experiment were isolated in plastic Petri dishes (Ø 9 cm × H 1.7 cm) on one leaf of their respective host plant in a climatic room at 23 ± 1°C, RH 65 ± 5% and photoperiod of 16:8 hr L:D. In order to increase the sample size for the performance analyses, additional replicates were performed under the same conditions as in part 1 without recording parasitoid behavior.

Parasitoid development within the host was monitored at four different times. The aphids were (a) dissected within 1 hr after being stung to check the presence of parasitoid eggs, under a binocular microscope at 100× magnification (to adjust the sting rate), (b) dissected after 4 days to measure survival of immature parasitoids under a binocular microscope at 40× magnification, and (c) checked at 7 days to monitor aphid mummification (number of replicate Table 1). The emergence rate of mummies and the sex ratio of emerged adults were recorded as well.

2.3 | Experiment 2: Presence of secondary endosymbionts in aphid

To evaluate the impact of aphid secondary endosymbionts on the development of juvenile parasitoids, each aphid colony was screened using PCR (Materials and Methods S1) to detect the presence of nine facultative symbiont genera (Table S1) that are known to interact with aphids (Desneux et al., 2018; Ferrari & Vavre, 2011): *Arsenophonus* sp., *Hamiltonella defensa* (T-type), PAXS (Pea-aphid X-type symbiont), *Regiella insecticola* (U-type), *Rickettsia* sp., *Rickettsiella* sp., *Serratia symbiotica* (R-type), *Spiroplasma* sp., *Wolbachia* sp (number of replicates for each aphid colony detailed Table 1). *Hamiltonella defensa* was found in all six *A. fabae* individuals tested and in 4 of the 6 *Metopolophium dirhodum* individuals tested. *Regiella insecticola* was found in all six *M. dirhodum* individuals tested. *Arsenophonus* sp., PAXS, *Rickettsia* sp., *Rickettsiella* sp., *Serratia symbiotica*, *Spiroplasma* sp., and *Wolbachia* sp. were not found in any of the aphid species screened.

2.4 | Data analysis

All statistical analyses were carried out using R version 3.2.2 (R Core Team 2017). The parasitoid behavior was analyzed using generalized linear models (GLMs) based on a binomial distribution. They were used (a) to compare parasitoid behaviors, i.e., detection, acceptance, and sting rates among aphid species, (b) to analyze the effect of aphid defensive behaviors on sting rate, and (c) to analyze the effect of aphid color, host plant, and aphid tribe on the proportion of aphids stung by the various parasitoids. The parasitoid performance was also analyzed using GLMs based on a binomial distribution. They were used (a) to compare proportion of egg, larvae, mummy, and

adult parasitoids recorded among all aphid species; (b) to compare parasitoid mortality across the different development stages in each aphid species, that is, egg, larvae, mummy, and adult stages; and (c) to analyze the effect of aphid color, host plant, and aphid tribe on the proportion of emerged parasitoids. When required, the GLMs were followed by a multicomparison test (Tukey, package "multcomp"). The deviation from a 0.5 sex ratio for only the parasitoids that produced ≥ 10 offspring (number enable to reasonably estimate a sex ratio) was tested with permuted Fisher's exact test (with the Bonferroni adjustment method). Finally, to analyze the relationship between the preference (sting rate) and the performance (emergence rate) of parasitoids, a comparative analysis of independent contrasts (calculation of phylogenetically independent variables, as described by Felsenstein (1985)) was used (CAIC, package "caper").

In addition to testing for occurrence of a PPH relationship, we also calculated the index of host specificity S_{TD}^* from Poulin and Mouillot (2005) to better characterize host specificity of tested parasitoids. Contrary to the former S_{TD} from Poulin and Mouillot (2003), the S_{TD}^* is a value which depends on (a) the prevalence of the parasitoid on the various hosts and (b) the position of these host species within a taxonomic hierarchy (Poulin & Mouillot, 2005). The smaller the value, the more specialist the parasitoid. The S_{TD}^* was computed using the program TaxoBiodiv2 (Poulin & Mouillot, 2005), which considers a taxonomic tree of hosts built based on family, tribe, genus, and species (Blackman & Eastop, 2006) and the proportions of parasitoid adult emergence for each species in every aphid species. It was done for the generalist parasitoids *A. abdominalis*, *A. ervi*, and *D. rapae*, as well as for three other parasitoid species previously analyzed in the laboratory: *Binodoxys communis* (Desneux, Barta, Hoelmer, et al., 2009) and *Binodoxys koreanus* (Desneux, Starý, et al., 2009) described as specialist aphid parasitoids as well as *Lysiphlebus testaceipes* described as moderate specialist aphid parasitoids (from France and from United States; Monticelli, 2018).

3 | RESULTS

For each parasitoid species tested, we tested the impact of the aphid colony, the host plant, the endosymbiont presence, the aphid color, and the aphid tribe on both the parasitoid preference and performance (Table 1).

3.1 | Parasitoid preference

For *Aphelinus abdominalis*, the proportion of aphid individuals detected by the parasitoid for each aphid species ranged from 0.91 to 1.00 and was not significantly different (Tables 2 and 3). Three aphid species, *A. craccivora*, *A. fabae* and *A. gossypii*, were significantly less accepted and stung by the parasitoid than *A. pisum*, *A. nerii*, *M. euphorbiae* (P and T), and *Sitobion avenae*. *Aphelinus abdominalis* stung less black than green aphids and stung mainly aphids from the Macrosiphini tribe. The presence of secondary endosymbiont or the different host plants tested did not impact their preference (Tables 3 and S2). Due

TABLE 2 Proportion of aphids detected, accepted, and stung by *Aphelinus abdominalis* (Aa), *Aphidius ervi* (Ae), and *Diaeretiella rapae* (Dr), respectively, upon the encounter of different aphid species

Aphid species/Parasitoid species	Proportion of aphids detected ^a			Proportion of aphids accepted			Proportion of aphids stung		
	Aa ^a	Ae	Dr	Aa	Ae	Dr	Aa	Ae	Dr
<i>Acyrtosiphon pisum</i>	1.00	1.00 a	1.00 a	0.97 a	0.95 a	0.83 ae	0.85 a	0.89 ab	0.65 ab
<i>Aphis craccivora</i>	1.00	1.00 a	0.58 c	0.48 b	0.5 b	0.19 cd	0.05 c	0.30 ce	0.13 c
<i>Aphis fabae</i>	0.94	0.92 b	0.83 bc	0.34 b	0.48 b	0.3 bcd	0.31 bc	0.29 ce	0.17 c
<i>Aphis gossypii</i>	0.91	0.96 b	0.93 b	0.34 b	0.77 ab	0.47 bde	0.25 bc	0.55 abcd	0.24 bc
<i>Aphis nerii</i>	1.00	0.98 ab	0.97 a	0.89 a	0.68 ab	0.74 ae	0.75 a	0.47 abcd	0.34 bc
<i>Brevicoryne brassicae</i>	0.97	0.96 b	1.00 a	0.73 ab	0.57 b	0.98 a	0.55 ab	0.10 e	0.87 a
<i>Macrosiphum euphorbiae</i> (P)	0.97	1.00 a	0.77 bc	0.94 a	0.96 a	0.43 bcde	0.88 a	0.86 a	0.14 c
<i>Macrosiphum euphorbiae</i> (T)	0.97	1.00 a	0.93 a	1.00 a	0.88 a	0.37 bcd	0.88 a	0.48 c	0.17 c
<i>Metopolophium dirhodum</i>	0.97	0.96 ab	0.77 bc	0.81 a	0.93 a	0.23 bc	0.69 ab	0.56 bc	0.13 c
<i>Myzus persicae</i> (Green)	0.97	0.97 ab	1.00 a	0.84 a	0.94 a	0.79 ae	0.64 ab	0.71 ad	0.64 ab
<i>Myzus persicae</i> (Red)	0.97	1.00 a	0.93 b	0.69 ab	0.93 a	0.63 ab	0.62 ab	0.55 abcd	0.39 bc
<i>Rhopalosiphum padi</i>	1.00	0.92 b	0.94 b	0.82 a	0.55 b	0.47 bcde	0.59 ab	0.28 ce	0.30 bc
<i>Schizaphis graminum</i>	0.97	0.91 b	0.98 ab	0.81 a	0.71 ab	0.95 a	0.63 ab	0.27 ce	0.76 a
<i>Sitobion avenae</i>	1.00	1.00 a	1.00 a	0.89 a	0.92 a	0.84 a	0.78 a	0.67 ad	0.54 abc

Note: For each parasitoid species, proportions followed by the same letter are not significantly different (GLMs followed by a multicomparison test) and the proportions of the rearing host (*A. pisum* for *A. abdominalis* and *A. ervi* and *B. brassicae* for *D. rapae*) are indicated in italics. The most detected, accepted, and stung aphid species are indicated in bold text.

^aNo significant difference in the proportion of aphids detected by *A. abdominalis* among the species.

to the very low number of *A. craccivora*, *A. fabae*, and *A. gossypii* stung by *A. abdominalis*, these aphid species could not be considered in the subsequent performance assessment.

For *Aphidius ervi*, the proportion of detected individuals of each aphid species ranged from 0.91 to 1.00 and varied significantly among the aphid species (Table 2 and 3). Five aphid species, *A. craccivora*, *A. fabae*, *B. brassicae*, *R. padi*, and *S. graminum*, were significantly less accepted and stung than *A. pisum*, *M. euphorbiae* (P), *M. persicae* (green strain), and *S. avenae*. *Aphidius ervi* accepted and stung less black than green aphids and aphids from the Aphidini tribe, regardless of the host plant species tested (Tables 3 and S2). *Brevicoryne brassicae* was stung by *A. ervi* at the lowest level and it could not be considered in the followed performance assessment.

For *D. rapae*, the proportion of aphid individuals detected ranged from 0.58 to 1.00 and varied significantly among the aphid species (Tables 2 and 3). Three aphid species (*A. craccivora*, *A. fabae*, and *M. dirhodum*) were significantly less accepted and stung than *A. pisum*, *B. brassicae*, *M. persicae* (green strain), and *S. graminum*. *Macrosiphum euphorbiae* (P and T) were not less accepted than these aphid species but were less stung. *Diaeretiella rapae* preference was similar regardless of aphid color, tribe, or host plant (Tables 3 and S2). Due to the very low number of *A. craccivora*, *A. fabae*, and *M. dirhodum* stung by *D. rapae*, these aphid species could not be considered in the subsequent performance assessment.

When attacked by *A. abdominalis*, *A. ervi*, and *D. rapae*, respectively 45%, 20%, and 62% of aphids exhibited defensive behaviors.

Acyrtosiphon pisum, *M. euphorbiae*, and *S. avenae* exhibited significantly higher defensive behaviors (57% rate of defensive reaction) than *A. craccivora*, *A. gossypii*, *A. nerii*, *M. dirhodum*, *M. persicae* (green strain), and *S. graminum* (34% rate of defensive reaction). There was a negative relationship between the proportion of aphids stung and exhibiting defensive behaviors when attacked by *A. abdominalis* ($\chi^2_1 = 53.1$, $p < 0.001$) (Figure 1). By contrast, this relationship was positive for *A. ervi* wasps ($\chi^2_1 = 53.1$, $p < 0.001$) and no relationship was observed for *D. rapae* ($\chi^2_1 = 3.3$, $p > 0.07$). The occurrence of aphid defensive behaviors thus varied depending on the parasitoid species ($\chi^2_1 = 270.9$, $p < 0.001$) and the aphid species ($\chi^2_{13} = 99.3$, $p < 0.001$).

3.2 | Parasitoid performance

For *Aphelinus abdominalis*, the offspring emergence rate ranged from 0.71 to 0.90, the highest being in *A. pisum*, *M. euphorbiae* (on potato and tomato plants), *M. dirhodum*, *M. persicae* (green and red strains), *R. padi*, and *S. avenae* (Table 3, Figure 2a). The other aphid species were grouped based on the stage at which the parasitoid development failed. First, in *Aphis nerii*, mortality was significantly higher between egg and larval stages ($\chi^2_3 = 63.0$, $p < 0.001$). Second, in *B. brassicae* and *S. graminum*, significant mortality was observed between larval and pupal stages ($\chi^2_3 = 48.7$ and 19.7, respectively, all $p < 0.001$). Finally, in *S. avenae*, mortality was observed during the pupal stage ($\chi^2_3 = 9.7$, $p = 0.022$). *Aphelinus abdominalis* performance

TABLE 3 Effect of the aphid colony, the host plant species, the presence of endosymbiont, the aphid color, and the aphid tribe on the parasitoid preference (proportion of aphids detected, accepted, and oviposited by the parasitoids) and the parasitoid performance (proportion of parasitoid larvae, of mummified aphid, and adult emerged). Significant effects of the various factors on the parasitoid preference and performance traits are indicated in bold text

	Parasitoid parameters (%)			Aphid colony			Plant species			Endosymbiont presence			Aphid color			Aphid tribe								
	Detection	Acceptance	Oviposition	χ^2	df	p value	Dp	χ^2	df	p value	Dp	χ^2	df	p value	Dp	χ^2	df	p value	Dp	χ^2	df	p value		
<i>Aphelinus abdominalis</i>	Detection	15.5	13	0.276	1.0	8.1	6	0.170	0.9	1.4	1	0.279	1.2	1.4	3	0.744	1.1	0.6	1	0.423	1.0	0.6	1	0.423
	Acceptance	112.5	13	<0.001	1.0	71.9	6	0.022	4.8	13.4	1	0.201	8.2	46.8	3	0.056	6.2	34.1	1	0.017	6.0	34.1	1	0.017
	Oviposition	111.3	13	<0.001	1.0	60.5	6	0.029	4.3	3.8	1	0.501	8.5	48.4	3	0.045	6.0	39.6	1	0.007	5.5	39.6	1	0.007
	Larvae	88.6	10	<0.001	1.0	73.3	5	<0.001	3.3	1.9	1	0.675	10.6	26.8	3	0.333	7.9	30.4	1	0.022	5.8	30.4	1	0.022
	Mummy	141.7	10	<0.001	1.0	87.8	5	0.132	10.4	3.2	1	0.633	14.2	9.5	3	0.913	18.0	40.5	1	0.063	11.7	40.5	1	0.063
Adult	123.9	10	<0.001	1.0	65.0	5	0.272	10.2	6.7	1	0.441	11.3	4.5	3	0.959	14.8	27.2	1	0.098	10.0	27.2	1	0.098	
<i>Aphidius ervi</i>	Detection	31.8	13	0.003	1.0	10.4	6	0.627	2.4	2.6	1	0.279	2.2	5.8	3	0.438	2.1	7.2	1	0.030	1.5	7.2	1	0.030
	Acceptance	155.9	13	<0.001	1.0	399	6	0.850	15.0	0.9	1	0.781	11.9	73.1	3	0.031	8.2	64.1	1	0.007	8.7	64.1	1	0.007
	Oviposition	196.9	13	<0.001	1.0	399	6	0.928	20.9	0.5	1	0.854	14.9	69.4	3	0.020	7.1	55.4	1	0.008	7.8	55.4	1	0.008
	Larvae	23.3	12	0.025	1.0	4.6	6	0.954	2.9	3.2	1	0.170	1.7	1.1	3	0.927	2.3	1.6	1	0.347	1.8	1.6	1	0.347
	Mummy	96.1	12	<0.001	1.0	10.9	6	0.992	13.8	24.4	1	0.039	5.7	60.6	3	<0.001	3.2	49.6	1	<0.001	3.8	49.6	1	<0.001
Adult	87.5	12	<0.001	1.0	14.8	6	0.974	11.9	19.3	1	0.071	5.9	51.5	3	0.002	3.4	38.7	1	0.001	3.7	38.7	1	0.001	
<i>Diaeretiella rapae</i>	Detection	72.1	13	<0.001	1.0	27.7	6	0.578	5.8	8.4	1	0.267	6.8	24.4	3	0.239	5.8	4.8	1	0.373	6.0	4.8	1	0.373
	Acceptance	157.7	13	<0.001	1.0	51.8	6	0.714	13.9	35.6	1	0.050	9.3	68.5	3	0.054	9.0	8.6	1	0.381	11.2	8.6	1	0.381
	Oviposition	129.9	13	<0.001	1.0	58.5	6	0.433	9.9	17.6	1	0.154	8.6	52.8	3	0.061	7.2	9.1	1	0.328	9.5	9.1	1	0.328
	Larvae	173.6	10	<0.001	1.0	173.5	6	<0.001	0.0	NA	NA	NA	NA	27.5	3	0.640	16.4	21.8	1	0.199	13.2	21.8	1	0.199
	Mummy	152.2	10	<0.001	1.0	125.0	6	<0.001	5.1	NA	NA	NA	NA	23.5	3	0.661	14.8	5.4	1	0.515	12.8	5.4	1	0.515
Adult	127.5	10	<0.001	1.0	103.9	6	<0.001	4.3	NA	NA	NA	NA	19.2	3	0.661	12.0	11.3	1	0.279	9.6	11.3	1	0.279	

Abbreviation: Dp, dispersion parameter.

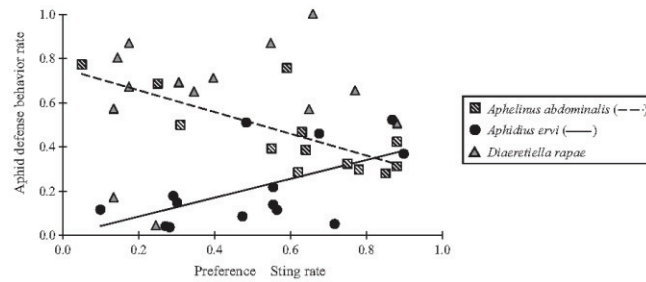


FIGURE 1 Relationship between the proportion of aphids stung in each aphid species by every parasitoid species tested (*Aphelinus abdominalis*, *Aphidius ervi*, and *Diaeretiella rapae*) and the proportion of aphid defensive behaviors exhibited by the aphid when it encountered these parasitoid species. The dashed line represents the negative relationship in *A. abdominalis* ($\chi^2_1 = 53.1$, $p < 0.001$), and the continuous line represents the positive relationship in *A. ervi* ($\chi^2_1 = 53.1$, $p < 0.001$). No relationship was observed in *D. rapae* ($\chi^2_1 = 3.3$, $p > 0.07$)

did not vary depending on the aphid colors and/or host plants (Tables 1, 3 and S2). However, a higher number of larvae survived in aphid species belonging to the Macrosiphini tribe (Table 3). The sex ratio was male-biased in *M. dirhodum*, *M. persicae* (green and red strains), and *R. padi* (all $p < 0.001$) and female-biased in *M. euphorbiae* on tomato ($p = 0.036$; Table 4).

For *Aphidius ervi*, the highest adult emergence rate was 0.62 and 0.69, respectively, for *S. avenae* and *A. pisum* (Table 3, Figure 2b). The other aphid species could be grouped upon the stage at which the parasitoid development failed. First, in *M. euphorbiae* on potato, mortality was significant higher between the egg and larval stages ($\chi^2_3 = 24.7$, $p < 0.001$). Second, in *A. craccivora*, *A. fabae*, *A. gossypii*, *A. nerii*, *M. dirhodum*, *R. padi*, and *S. graminum*, significant mortality was observed between the larval and pupal stages ($\chi^2_3 > 15.0$, respectively, all $p < 0.001$). Finally, in *M. persicae* (green and red strains), mortality was primarily observed during the pupal stage ($\chi^2_3 = 17.8$, $p < 0.001$). *Aphidius ervi* produced a higher proportion of offspring in the green aphids compared to the black ones, in the aphids belonging to the Macrosiphini tribe and in the aphids that did not harbor a secondary endosymbiont (Tables 1 and 3). The host plant did not modulate the performance of *A. ervi* (Tables 1, 3, and S2). The *A. ervi* sex ratio was similar to 50:50 in all aphid species tested (all $p > 0.05$; Table 4).

For *D. rapae*, the adult emergence rate ranged from 0.38 to 0.61 and the highest was in *A. gossypii*, *B. brassicae*, *M. persicae* (green and red strains), *R. padi*, and *S. avenae* (Table 3, Figure 2c). The other aphid species could be grouped upon the stage at which parasitoid development failed. First, mortality was significant between the egg and larval stages in *A. pisum* and *M. euphorbiae* ($\chi^2_3 = 63.9$, 58.8, all $p < 0.001$). Second, significant mortality was observed between the larval and pupal stages in *A. nerii* and *S. graminum* ($\chi^2_3 = 83.9$ and 72.0, respectively, all $p < 0.001$). Finally, mortality was observed during the pupal stage in *B. brassicae*, *M. persicae* (green strain), and *R. padi* ($\chi^2_3 = 13.9$, 16.3, and 11.9, $p = 0.003$, 0.0009, and 0.007, respectively). *Diaeretiella rapae* performance was modulated by the host plant, and a lower offspring proportion was observed in aphid species maintained

on *Asclepias*, bean, potato, and tomato than on cabbage, squash, and wheat (Tables 1, 3, and S2). However, the aphid color and tribe did not modulate the parasitoid performance (Tables 1 and 3). The *D. rapae* sex ratio was similar to 50:50 in all aphid species (all $p > 0.05$; Table 3).

3.3 | The PPH relationship

The preference–performance hypothesis (PPH) have been found in specialist arthropod, but it has been argued that such relationships may not be common in generalist ones. In this study, no significant relationship was found between the preference (sting rate) and the performance (emergence rate) of *A. abdominalis* ($F_{1,7} = 0.99$, $p = 0.353$, $R^2 = 0.12$), *A. ervi* ($F_{1,9} = 3.96$, $p = 0.078$, $R^2 = 0.31$), and *D. rapae* ($F_{1,7} = 0.21$, $p = 0.663$, $R^2 = 0.03$; Figures 3 and S1).

3.4 | Index of host specificity

Aphelinus abdominalis and *D. rapae* are the more generalist aphid parasitoid considered in this study and their S_{TD}^* are 2.37 and 2.55, respectively (Figure 4). *Aphidius ervi* and *L. testaceipes* (from previous study) are oligophagous species and their S_{TD}^* are 2.21, 1.75, and 1.83, respectively. Finally, *B. communis* and *B. koreanus* (from previous study) were the most specialized species considered in our study and their S_{TD}^* are, respectively, 1.17 and 1.32.

4 | DISCUSSION

Generalist parasitoids are known to use a broad set of cues to identify potential hosts, and this may alter the relationship between their preference and performance traits (Mackauer et al., 1996; Vet & Dicke, 1992). Testing such a PPH relationship in three generalist aphid parasitoids on twelve aphid host species revealed that such a relationship is absent in *A. abdominalis*, *A. ervi*, and *D. rapae*. All three parasitoids showed low behavioral selectivity when encountering

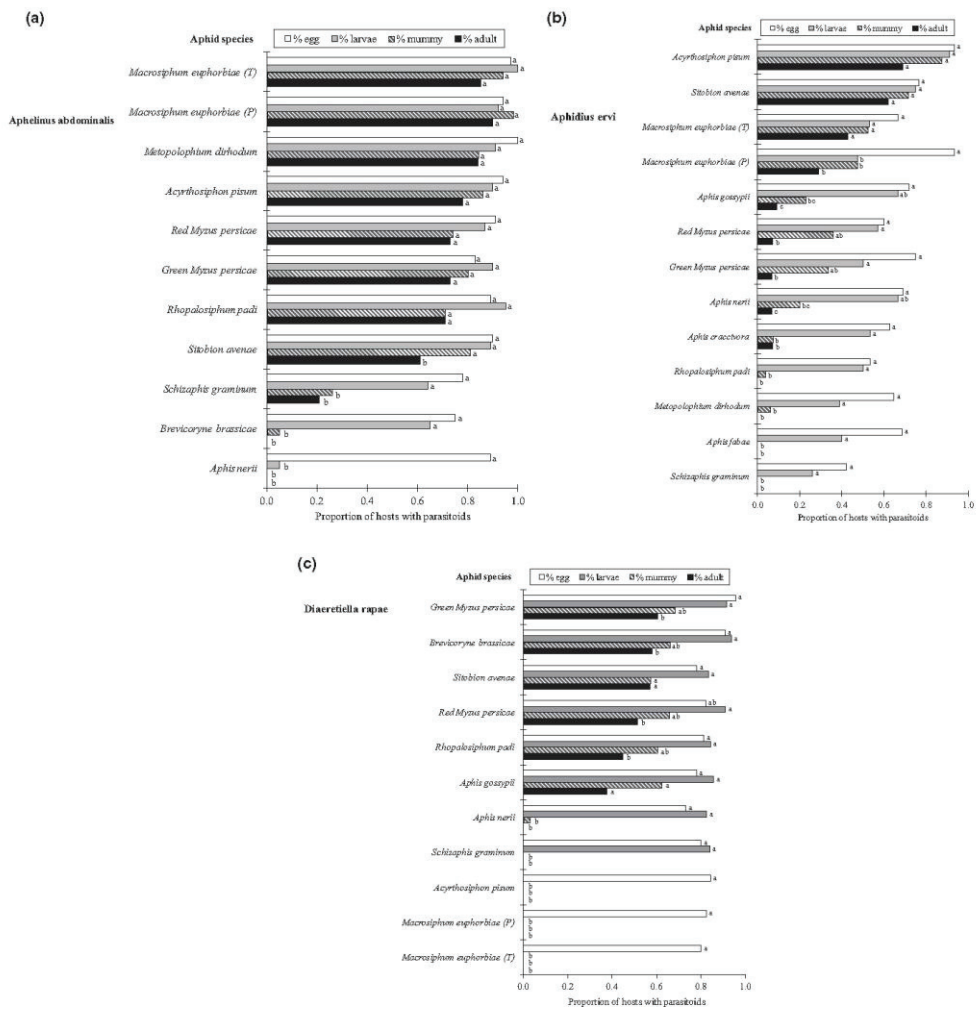


FIGURE 2 Proportion of stinging aphids that contained an egg (dissection after stung), contained a larva (dissection after 4 days), mummified (after 10 days), and produced an adult parasitoid for (a) *Aphelinus abdominalis*, (b) *Aphidius ervi*, and (c) *Diaeretiella rapae* (experiment 2). For each aphid species, bars followed by the same letter are not significantly different (generalized linear models followed by multicomparison test)

potential host species (preference traits), and thus, their host range was primarily dictated by the actual host suitability for their offspring development. Host suitability in two particular aphid species was likely affected by the presence of two detected secondary endosymbionts (von Burg, Ferrari, Muller, & Vorburger, 2008; Oliver et al., 2003). In parallel, the calculated S_{TD}^* values were consistent with the results from PPH assessments (present study and our previous ones) and enabled the categorization of the aphid parasitoids as generalist

(*D. rapae* and *A. abdominalis*), oligophagous (*A. ervi* and *L. testaceipes*), or more specialized (*B. communis* and *B. koreanus*) species.

4.1 | Preference traits

As expected for generalist parasitoids, the three species tested showed low host selectivity, i.e., they stung all aphid species encountered (at least in some extent) regardless to the host plant species

Aphid species	Female sex ratio (proportion females)		
	<i>Aphelinus abdominalis</i>	<i>Aphidius ervi</i>	<i>Diaeretiella rapae</i>
<i>Acyrtosiphon pisum</i>	0.67	0.73	n/a
<i>Aphis gossypii</i>	n/a	n/a	n/a
<i>Brevicoryne brassicae</i>	n/a	n/a	0.59
<i>Macrosiphum euphorbiae</i> (P)	0.44	0.60	n/a
<i>Macrosiphum euphorbiae</i> (T)	0.76*	0.56	n/a
<i>Metopolophium dirhodum</i>	0.04***	n/a	n/a
<i>Myzus persicae</i> (red)	0***	n/a	0.50
<i>Myzus persicae</i> (green)	0.09***	n/a	0.61
<i>Rhopalosiphum padi</i>	0.05***	n/a	0.59
<i>Sitobion avenae</i>	0.32	0.54	0.55
<i>Aphis fabae</i>	n/a	n/a	n/a
<i>Aphis craccivora</i>	n/a	n/a	n/a
<i>Schizaphis graminum</i>	n/a	n/a	n/a
<i>Aphis nerii</i>	n/a	n/a	n/a

Note: n/a means that data are nonavailable due to the unsuccessful development of the parasitoids in these aphid species.

* $p < 0.05$,

*** $p < 0.001$ (deviation from a 0.5 sex ratio).

or the endosymbiont presence. However, *A. abdominalis* and *A. ervi* might show a preference for the green aphids over the black ones (though confounding effect could not be excluded, for example, all Macrosiphini aphids tested were greenish, whereas all the black aphids belonged to the Aphidini tribe). Still, visual cues are used by many organisms to evaluate and select resources (Bell, 1991), and green aphids are known to be well detected by aphid parasitoids; *Aphidius rhopalosiphii*, *Monoctonus paulensis*, and *Praon pequodorum* preferred green aphids (Michaud & Mackauer, 1994, 1995). In addition, *A. abdominalis* and *A. ervi* attacked significantly more aphids from the Macrosiphini tribe. Overall, they stung all the aphid species from the Macrosiphini tribe encountered at high proportions and some species from the Aphidini tribe, such as *R. padi* and *S. graminum*. Closely related species may share characteristics recognized by parasitoid to select their hosts (Bell, 1991; Harvey et al., 1991; Ives & Godfray, 2006; Michaud & Mackauer, 1994). In our study, we did not consider the beginning of the host selection process which may occur at long distance, i.e., habitat location (according to the definition by Vinson, 1985) and doing so we could have missed a part of the behavioral selectivity. However, the host selection process by generalist parasitoids mostly relies on semiochemicals originating from the hosts themselves (Becker et al., 2015) and notably in aphid parasitoids (Hatano, Kunert, Michaud, & Weisser, 2008).

Aphid defensive behaviors are known to potentially affect oviposition behavior of various aphid parasitoids and to reduce the parasitoid host range (Desneux, Barta, Hoelmer, et al., 2009; Kouamé & Mackauer, 1991; Wyckhuys et al., 2008). To avoid

TABLE 4 Female sex ratio (proportions: females/adult emerged) for each parasitoid species developing on different hosts.

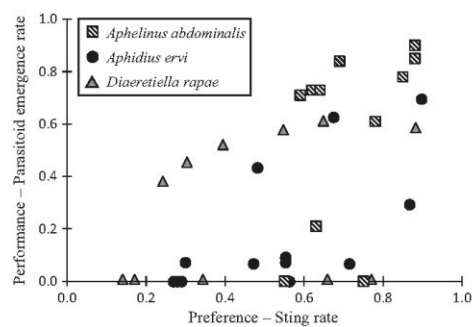


FIGURE 3 Relationship between the sting rate (preference) and the emergence rate (performance) when three generalist parasitoids (*Aphelinus abdominalis*, *Aphidius ervi*, and *Diaeretiella rapae*) encountered twelve aphid species. Relationship between the proportion of aphids stung in each aphid species by every parasitoid species tested (*A. abdominalis*, *A. ervi*, and *D. rapae*) and the proportion of emerged adults of the aphids stung. No relationship was observed in *A. abdominalis* ($F_{1,7} = 0.99$, $p = 0.353$, $R^2 = 0.12$), *A. ervi* ($F_{1,9} = 3.96$, $p = 0.078$, $R^2 = 0.31$), and *D. rapae* ($F_{1,7} = 0.21$, $p = 0.663$, $R^2 = 0.03$)

aphid defensive behaviors, some parasitoids, including *A. ervi* and *D. rapae*, exhibited a "quick" sting syndrome (Desneux, Barta, Delebecque, & Heimpel, 2009; Völkl & Mackauer, 2000). In this

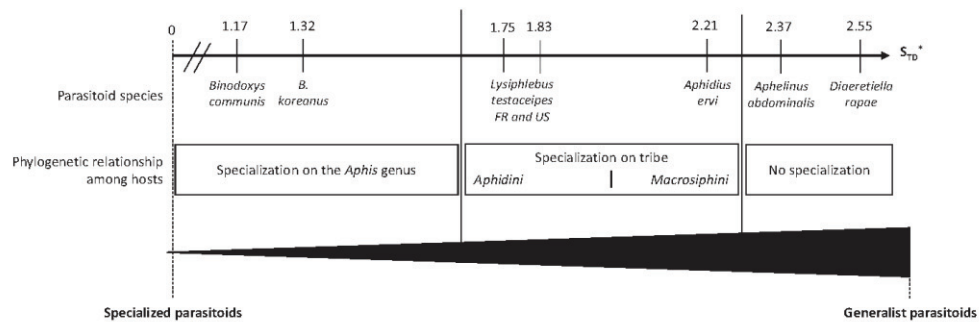


FIGURE 4 Host specificity index (S_{TD}^*) values and ranking of the parasitoids according to their degree of specialization

study, *D. rapae* did not show a relationship between its sting rate and the aphid defense rate, suggesting that *D. rapae* is able to avoid aphid defensive behaviors. A positive relationship was found between the *A. ervi* sting rate and the aphid defenses rate induced by this parasitoid species; aphid defenses such as cornicle secretions and defensive movements might be used by *A. ervi* as chemical and physical cues, respectively (Battaglia et al., 2000). Contrarily, there was a negative relationship between the proportion of aphid species stung by *A. abdominalis* and the proportion of aphid defensive behaviors. *Aphelinus abdominalis* demonstrated a sting time ranging between 20 and 60 s, about four times that of the two other parasitoid species tested. This suggests that aphid defensive behaviors may disturb females during their stinging event (De Farias & Hopper, 1999; Wahab, 1985). Secondly, success of aphid defenses depends on the relative size of the attacking parasitoid versus the aphid. *Aphelinus abdominalis* is two times smaller than *A. ervi* and *D. rapae*, whose size allows them to attack aphids more easily (Le Ralec et al., 2010).

4.2 | Performance traits

Aphelinus abdominalis and *D. rapae* were able to produce offspring with high prevalence in aphids from both Aphidini and Macrosiphini tribes, whereas *A. ervi* was able to produce offspring in aphid species mainly from the Macrosiphini. As previously, closely related species can share characteristics used by parasitoids to complete their development in their hosts (Harvey et Pagel, 1991; Ives et Godfray, 2006), suggesting that *A. ervi* is specialized on aphids belonging to the Macrosiphini tribe (as in Zepeda-Paulo, Ortiz-Martínez, Figueroa, & Lavandero, 2013).

Different physiological and ecological factors could then provide aphids with resistance against immature parasitoids and could modify parasitoid host range (Monticelli, 2018). The main sources of resistance include poor parasitoid ability to control host metabolism (Godfray, 1994), the aphid host plant (Desneux, Barta, Hoelmer, et al., 2009), the presence of secondary endosymbionts (Oliver, Moran, & Hunter, 2005; Vorburger, Gehr, & Rodríguez, 2009), the aphids'

ability to sequester toxic compounds (Desneux, Barta, Hoelmer, et al., 2009; Francis, Lognay, Wathélet, & Haubruge, 2001), and/or the host quality itself (Godfray, 1994; Kouamé & Mackauer, 1991).

The facultative endosymbionts present in aphids may compromise the successful development of parasitoids explaining the parasitoid mortality between the egg and larvae stage (Ferrari, Darby, Daniell, Godfray, & Douglas, 2004; McLean & Godfray, 2015; Oliver et al., 2005). Specifically, *Hamiltonella defensa* and *Regiella insecticola* (detected in *A. fabae* and *M. dirhodum* as reported by Henry, Maiden, Ferrari, & Godfray, 2015) associated with a toxin-encoding bacteriophage (ASPE, Oliver, Degnan, Hunter, & Moran, 2009) are known to provide aphids protection against different natural enemies, such as parasitoids (Oliver et al., 2003). In this study, *A. abdominalis* was not impacted by the presence of secondary endosymbionts since it had 84% successful parasitism in *M. dirhodum* (as in McLean, Hrček, Parker, & Godfray, 2017 and Hopper et al., 2018). The presence of *R. insecticola* may, in the case of *Aphelinus* sp., induce a higher parasitism rate (Luo et al., 2017) or a higher fitness, for example, *Aphelinus glycinis* produced more and larger female adult progeny on infected than on uninfected aphids (Hopper et al., 2018). By contrast, a strong parasitoid larval mortality of *Aphidius ervi* was observed when it encountered *A. fabae* and *M. dirhodum*, suggesting that *H. defensa* and/or *R. insecticola* had a strong negative impact on *A. ervi* performance (as in Oliver et al., 2003, Vorburger et al., 2009). In addition, *A. ervi* is well known to parasitize Macrosiphini species such as *M. dirhodum* (Starý 1993, Kavallieratos et al., 2004), suggesting that the presence of *H. defensa* and/or *R. insecticola* reduces the *A. ervi* host range. Finally, *D. rapae* stung *A. fabae* and *M. dirhodum* at a low rate that does not permit an evaluation of the impact of *H. defensa* and *R. insecticola* on parasitoid performance. The impact of endosymbionts is variable depending on the aphid-parasitoid system considered and more studies are needed. Several studies have shown that endosymbionts confer protection only against the more specialized natural enemies and less against generalist ones (Asplen et al., 2014; Hrček, McLean, & Godfray, 2016; Kraft, Kopco, Harmon, & Oliver, 2017; Parker, Spragg, Altincicek, & Gerardo, 2013), which support the hypothesis whereby *A. ervi* is more specialized than *A. abdominalis* and *D. rapae*.

The host plant may also contribute to a reduction in aphid parasitoid host range. *Aphelinus abdominalis* and *A. ervi* can parasitize aphids on multiple host plants, whereas *D. rapae* fails to develop in aphid species maintained on milkweed, bean, potato, and tomato. *Diaeretiella rapae* is an aphid parasitoid generalist and a habitat specialist (notably on Brassicaceae and Gramineae, Kavallieratos et al., 2004). In our study, *A. craccivora* and *A. fabae* were not stung by *D. rapae*, whereas when these aphid species are found on Brassicaceae, they are considered as suitable hosts for *D. rapae* (Alikhani, Rezvani, Starý, Kavallieratos, & Rakhshani, 2013; Kavallieratos et al., 2004), suggesting that bean modulates parasitoid performance.

The specialist aphid species *A. nerii* and *B. brassicae* are able to sequester cardenolide (*Asclepias*) and glucosinolate (cabbage), respectively (Desneux, Barta, Hoelmer, et al., 2009; Jones, Bridges, Bones, Cole, & Rossiter, 2001), and these toxic allelochemical molecules have a drastic impact on immature parasitoid survival (Desneux, Barta, Hoelmer, et al., 2009; Kos et al., 2012; Mooney, Jones, & Agrawal, 2008; Pratt, Pope, Powell, & Rossiter, 2008) and may explain the parasitoid mortality between the larvae and pupae stage. The three generalist parasitoids cannot successfully parasitize *A. nerii* aphids living on *Asclepias*, despite a high sting rate (up to 0.75) equal to that of other, nontoxic host plants. All developing parasitoids reached the larval stage and then died. *Diaeretiella rapae* is able to use glucosinolates (and/or related compounds) as long- and short-distance kairomones for selecting its hosts (Bradburne & Mithen, 2000) as well as to promote effective development of its offspring in aphids such as *B. brassicae* (Kos et al., 2012). However, *A. abdominalis* cannot successfully parasitize *B. brassicae* despite a non-negligible sting rate (0.55). *Myzus persicae* also feeds on cabbage but is not a glucosinolate-sequestering aphid and excretes the glucosinolates in its honeydew, reducing the impact on parasitoid offspring development (Francis et al., 2001; Weber 1986). *Aphelinus abdominalis* exhibits successful development in *M. persicae* (0.73 adults emerged), suggesting that *A. abdominalis* is strongly affected by glucosinolates. Conversely, *A. ervi* did not sting *B. brassicae* and cannot complete its development in *M. persicae*, which is generally found to be a suitable host (Colinet, Salin, Boivin, & Hance, 2005; Kavallieratos et al., 2004), suggesting that aphid genotype could be involved in this failure to parasitize (Bilodeau, Simon, Guay, Turgeon, & Cloutier, 2013; von Burg et al., 2008). Aphid ability to sequester the toxic compounds from their host plant involves a high specialization of aphid species (Mooney et al., 2008) and only a few (up to four) parasitoid species can parasitize *A. nerii* and *B. brassicae* (Kavallieratos et al., 2004), suggesting that strong circumventing mechanisms are needed for a parasitoid to adapt to aphid defense. Furthermore, sequestering is a general aphid defense against parasitoids as well as natural enemies (Omkar & Mishra, 2005; Toft & Wise, 1999).

Finally, host quality could also contribute to a high mortality of later parasitoid larval stages prior to emergence, or at least a modulation of their sex ratio (male-biased; Godfray, 1994; Kouamé & Mackauer, 1991; Mackauer, 1986). Host species and age are the two most important factors determining parasitoid development. However, generalist parasitoids are less demanding in terms of host choice, as shown in the behavioral results of this study. For example, *S. graminum* caused a high larval mortality of the three parasitoid

species (consistent with Desneux, Barta, Hoelmer, et al., 2009) and a high proportion of unemerged parasitoids of *A. ervi* were observed in *R. padi* despite high sting rates, indicating that *S. graminum* and *R. padi* are poor hosts for these parasitoid species. Furthermore, some parasitoids tend to place male eggs in unfavorable hosts (Godfray, 1994; Kochetova, 1978). In *A. abdominalis*, a male-biased sex ratio was observed in *M. dirhodum*, *M. persicae* (green and red strains), *R. padi*, and *S. graminum*, suggesting that these aphid species are considered as low quality hosts for *A. abdominalis*.

4.3 | The preference–performance relationship in generalist parasitoids

The meta-analysis of Gripenberg et al. (2010) of the PPH relationship in phytophagous insects described a relationship between preference and the performance, present in specialist, but lacking in generalists. The lack of the PPH relationship in *A. abdominalis* and *D. rapae* is due to their low host selectivity and their high performance in multiple hosts (in 6 and 5 aphid species, respectively). In *A. ervi*, both its preference and performance were significantly higher in the aphids belonging to the Macrosiphini tribe (as in Zepeda-Paulo et al., 2013), suggesting a host phylogenetic specialization (Desneux, Blahnik, Delebecque, & Heimpel, 2012). The PPH relationship provides useful clues to classify parasitoids in terms of degree of specialization, although it does not enable strictly separating generalist from oligophagous organisms. Hence, the host specificity index (from Poulin & Mouillot, 2005) provides useful complementary information, quantifying where these species lie on a generalist–specialist continuum. Indeed, when comparing the S_{TD}^* values of the parasitoids tested in this study and the parasitoids tested in our previous studies, we demonstrated that *A. abdominalis* and *D. rapae* are generalist aphid parasitoids and are able to produce offspring with high prevalence in aphids from both Aphidini and Macrosiphini tribes, whereas *A. ervi* and *L. testaceipes* are moderate specialist aphid parasitoids able producing offspring in aphid species mainly from the Macrosiphini or Aphidini tribe, respectively. Finally, *B. communis* and *B. koreanus* are classified as specialist parasitoids, being able to produce offspring mainly in aphids belonging to the *Aphis* genus (with the exception of *S. graminum*, an aphid species from the Aphidini tribe, still closely related to the *Aphis* genus, Desneux, Barta, Hoelmer, et al., 2009; Desneux, Starý, et al., 2009).

Parasitoid within-species genetic variability could have some degree of influence on preference- and/or performance- related traits (Cayetano & Vorburger, 2015; Diehl & Bush, 1984; Raymond, Plantegenest, Gagic, Navasse, & Lavandero, 2015). For example, Derocles et al. (2016) reported that various generalist parasitoid species are composed of biotypes linked to a given host species. This suggests that results on behavioral and/or physiological traits involved in parasitoid specialization might vary slightly according to the actual biotype considered when studying a given parasitoid species. For example, a biotype of *D. rapae* reared on *M. persicae* parasitized only *M. persicae* and *B. brassicae*, whereas another biotype reared on *Hayhurstia atriplicis* was able to parasitize *H. atriplicis*, *M. persicae*, *B. brassicae*, and *A. fabae* (Navasse, Derocles, Plantegenest, & Ralec,

2018). Therefore, examining several biotypes of a same parasitoid species may be useful when characterizing its degree of specialization. It may also help to provide a more accurate assessment when developing biological control programs requiring parasitoids with a high degree of specialization (e.g., classical biological control).

5 | CONCLUSION

We demonstrated that the preference–performance relationship is present for specialist parasitoids, but not for intermediate specialist–generalist and true generalists, likely owing to combined effects of low selectivity and variable performance in generalist parasitoids (van Klinken, 2000). The generalists are less affected by specific aphid defenses against them (such as endosymbionts, whereas they are strongly affected by general ones that are used against natural enemies (e.g., aphid ability to sequester the toxic compounds). The preference of generalists is not an accurate proxy of actual parasitoid realized host range, i.e., performance. The occurrence (or lack thereof) of such a relationship, as well as the host specificity index, may provide a reliable indicator of actual generalism–specialism in parasitoids.

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CONFLICT OF INTEREST

None declared.

DATA ACCESSIBILITY

Data for this study are available at [datadryad.org/](https://doi.org/10.5061/dryad.n88s5r3) (<https://doi.org/10.5061/dryad.n88s5r3>) (Monticelli et., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary material.

Supplementary methods 1.

DNA extraction. Each sampling was washed in 70 % ethanol for 2 to 5 minutes (aphid size dependent), rinsed in PBS (phosphate buffer solution) for 1 minute and finally washed in pure water. Samples were homogenized with piston (1 piston/sample) in Lysis buffer for DNA extraction. Then, samples were placed in 10 μ L of RNase A, 50 μ L of lysozyme and 20 μ L of protease K and incubated at 55 °C for 3 hours. For DNA purification, samples were centrifuged at 3000 rpm for 5 minutes and the supernatants were collected. 1 mL of absolute ethanol and 50 μ L of sodium acetate were added and the mixture was placed at -20°C overnight. Samples were then centrifuged at 14000 rpm for 20 minutes and the supernatants were removed. After adding 1 mL of 70 % ethanol, the mixture was homogenized and centrifuged at 14000 rpm for 5 minutes. After 5 minutes at room temperature to let the pellet dry, 50 μ L of pure water was added to the pellet and then stored at -20 °C until used. The quantity and quality of the DNA were measured using NanoDrop and diluted to obtain 50 ng of DNA / μ L for each sample.

PCR amplification. Diagnostic PCR reactions with symbiont-specific primer pairs (Sup table 1) were conducted in 1.5 % agarose gel stained with ethidium bromide and visualized with UV light to test the presence of facultative symbionts. Each symbiont was tested with a positive control (DNA from aphid species) and two negative controls (two pea aphid genotypes that were known to harbor only the primary endosymbiont *Buchnera aphidicola*). Furthermore, the quality of the extraction was tested by PCR on the primary endosymbiont: *B. aphidicola*.

Finally, bands showing a signal were removed from the gel, purified (with Min Elute PCR purification kit) and sequenced to check the symbiont identity (validated when the sequence was at least 95% similar).

Supplementary table T1. Endosymbiont targeted, target gene, primer name and sequences used to detect the symbionts present in the different aphid species.

Target	Target gene	Primer name	Sequences
<i>Hamiltonella defensa</i> (T-type)	16S rDNA	16S-8F 16S-480R	AGAGTTTGATCMTGGCTCAG GGTATTCGCATTTATCGCTTC
<i>Buchnera</i>	DnaK	BuchDnaK 12F BuchDnaK 162R	TATTGGTATTGACTTGGGAA AGCAGGTTGTCTACTAAAAC
<i>Regiella insecticola</i> (U-type)	16S rDNA	16S-8F 16S-R2	AGAGTTTGATCMTGGCTCAG TCGGACGCCATAA CACTAGG
<i>Rickettsiella</i>	16S rDNA	P136F-16S P136Ric-470R-16S	GGGCCTTGCCTCTAGGT TGGGTACCGTCACAGTAATCGA
PAXS	16S rDNA	PAXS F-16S PAXS R-16S	AGTTTGATCATGGCTCAGATTG GCAACACTCTTTCATTGCT
<i>Serratia symbiotica</i> (R-type)	16S rDNA	16S-8F PASS1140R	AGAGTTTGATCMTGGCTCAG TTTGAGTTCCCGACTTTATCG
<i>Rickettsia</i>	16S rDNA	ricCsA-318-F-GltA ricCsA-318-R-GltA	TGATCATGAGCAAAATGCTT TCTAGCTGCCCAAGTCTTT
<i>Spiroplasma</i>	16S rDNA	Spi 618834-F-16S Spi 618834-R	GTGGCAAGCGTTATCCGGAT CCCACGCTTTCGTGCCACAA
<i>Wolbachia</i>	FtsZ	Wol-FtsZ-F Wol-FtsZ-R	TTGCAGAGCTTGGACTTGAA CATATCTCCGCCACCAGTAA
<i>Arsenophonus</i>	yaeT	Ars-yaeT-F Ars-yaeT-R	GCATACGGTTCAGACGGGTTTG GCCGAAACGCCTTCAGAAAAG

Supplementary table T2. Proportion of aphids detected, accepted, and stung by parasitoids as well as the proportion of egg, larvae, mummy and adult *Aphelinus abdominalis*, *Aphidius ervi* and *Diaeretiella rapae* upon the encounter of different host plant species.

	Host plants	Parasitoid preference			Parasitoid performance			
		% Detected ^a	% Accepted ^a	% Stung ^a	% Egg ^a	% Larvae ^a	% Mummy ^a	% Adult ^a
<i>Aphelinus abdominalis</i>	Potato	0.97	0.94	0.88	0.94	0.92	0.98	0.9
	Tomato	1	1	0.88	0.97	1	0.94	0.85
	Bean	0.98	0.59	0.4	0.94	0.9	0.86	0.78
	Wheat	0.99	0.83	0.67	0.89	0.85	0.66	0.59
	Cabbage	0.97	0.75	0.6	0.83	0.81	0.53	0.49
	Asclepias	1	0.89	0.75	0.89	0.05	0	0
	Squash	0.91	0.34	0.25	0	0	0	0
<i>Aphidius ervi</i>	Tomato	1	0.88	0.48	0.67	0.53	0.52	0.43
	Potato	1	0.96	0.86	0.93	0.48	0.47	0.29
	Bean	0.97	0.64	0.49	0.75	0.61	0.32	0.25
	Wheat	0.95	0.76	0.45	0.59	0.47	0.2	0.15
	Squash	0.96	0.77	0.55	0.72	0.67	0.23	0.09
	Cabbage	0.98	0.81	0.45	0.68	0.54	0.35	0.07
	Asclepias	0.98	0.68	0.47	0.69	0.67	0.2	0.07
<i>Diaeretiella rapae</i>	Cabbage	0.98	0.8	0.63	0.86	0.93 ^a	0.66 ^a	0.57 ^a
	Squash	0.93	0.47	0.24	0.67	0.86 ^a	0.63 ^a	0.38 ^a
	Wheat	0.92	0.62	0.45	0.78	0.84 ^a	0.39 ^a	0.34 ^a
	Asclepias	0.97	0.74	0.34	0.62	0.83 ^a	0.03 ^b	0 ^b
	Potato	0.77	0.43	0.14	0.82	0 ^b	0 ^b	0 ^b
	Tomato	0.93	0.37	0.17	0.82	0 ^b	0 ^b	0 ^b
	Bean	0.8	0.44	0.31	0.84	0 ^b	0 ^b	0 ^b

For each parasitoid species, proportions followed by the same letter are not significantly different (GLMs followed by a multi comparison test). ^a No significant difference in the proportion of aphids detected among species for each parasitoid species tested.

Supplementary figure S1. Relationship between the stinging rate (preference) and the emergence rate (performance) of three generalist aphid parasitoids (*Aphelinus abdominalis* (A), *Aphidius ervi* (B) and *Diaeretiella rapae* (C) when encountering twelve different aphid species.

Figure S1. A. *Aphelinus abdominalis*

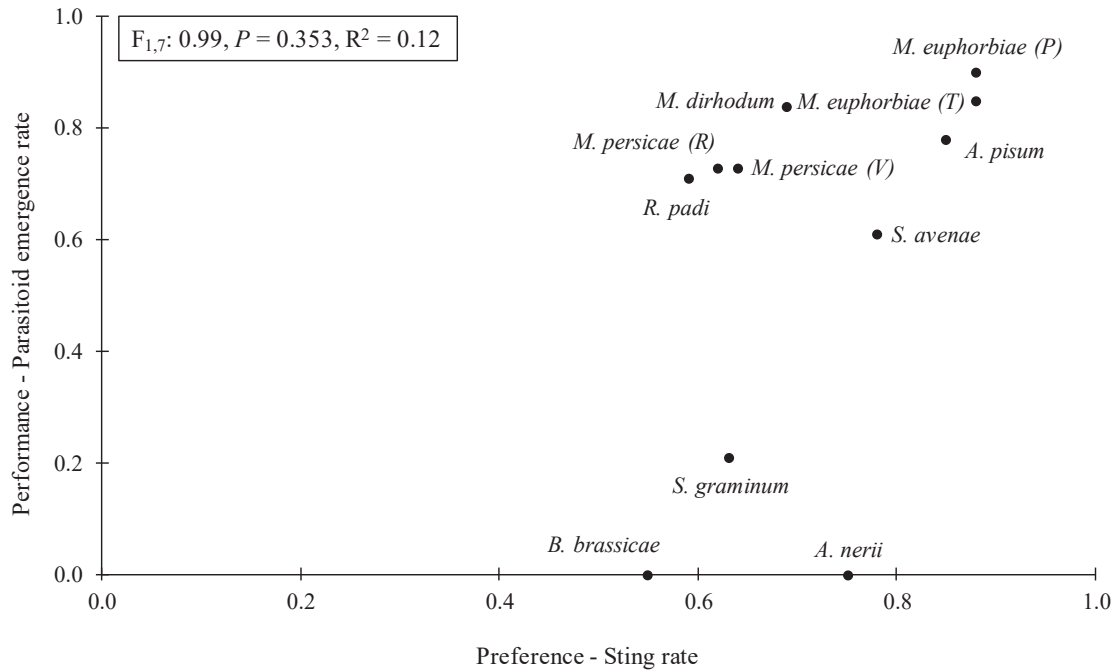


Figure S1. B. *Aphidius ervi*

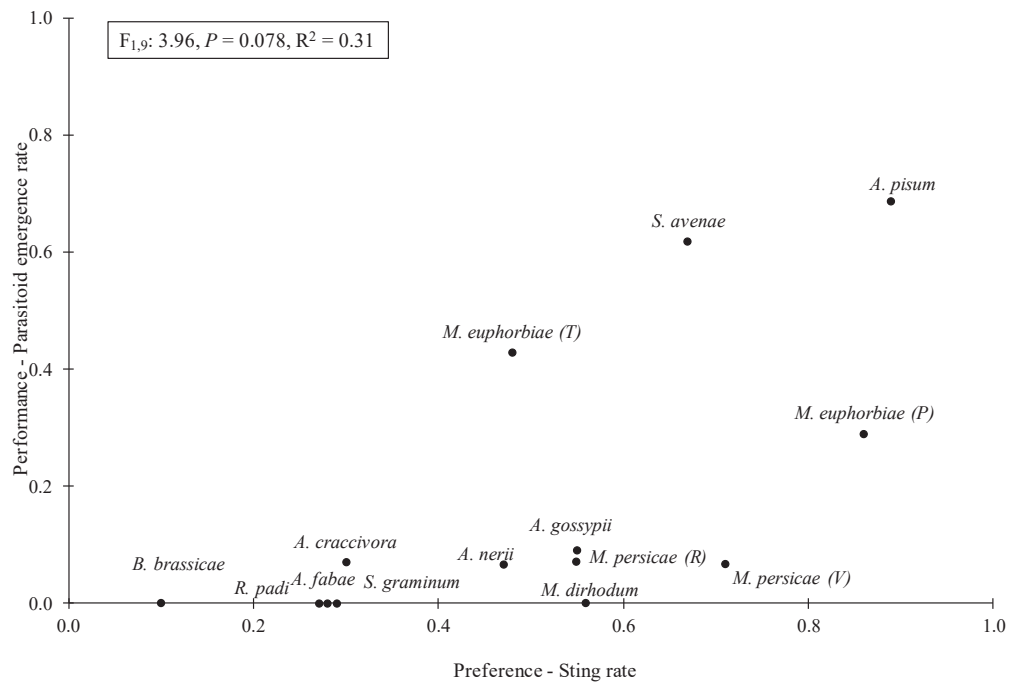
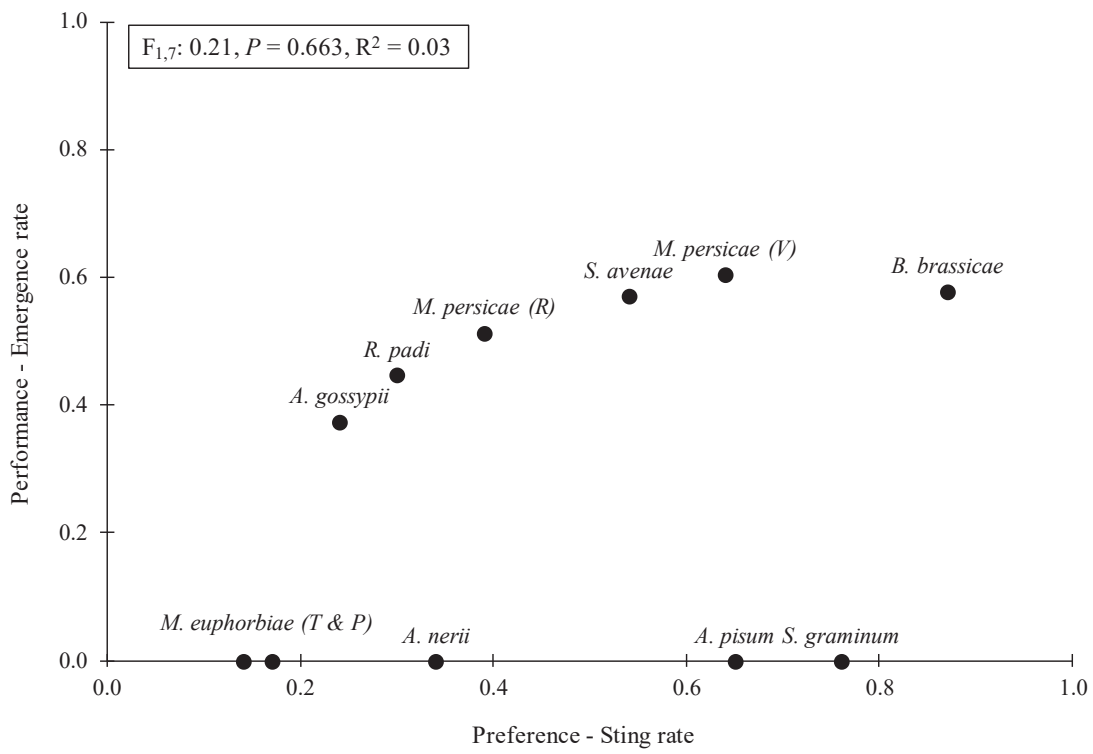


Figure S1. C. *Diaeretiella rapae*



Conclusion and perspectives of publication 2

The three parasitoids tested attacked all introduced aphid species, confirming their status as a generalist. However, only 42 to 58% of the aphid species attacked allowed the development and production of parasitoid offspring. The preference and performance were correlated for *A. ervi* but not *A. abdominalis* and *D. rapae*. *A. ervi* develops best in hosts belonging to the Macrosiphini tribe, suggesting that this parasitoid is not a true generalist parasitoid (able to attack hosts of different tribes) but rather an oligophagous species, with a restricted range of host species. Thus, a positive relationship between parasitoid preferences and performance seems to exist in some extent in oligophagous parasitoids, but not in generalists. In our study, the presence of secondary endosymbiont or the different host plants tested had not significant impact on the parasitoid preference, although it did have some on the success of *A. ervi*. High larval parasitoid mortality was observed on *A. fabae* and *M. dirhodum*, suggesting that *H. defensa* and/or *R. insecticola* had a strong negative impact on the performance of *A. ervi*.

The parasitoid host specificity index was also calculated as described by Poulin and Mouillot (2005). This index assigns value to each parasitoid based on the phylogenetic relationships between their hosts and their rate of development in these hosts. It was calculated for the three parasitoids but also for three other parasitoids previously tested and considered specialists (*Binodoxys communis*, *B. koreanus* and *Lysiphlebus testaceipes*) (Braconidae) (Desneux et al. 2009 a, b). The computation of this index allowed to rank these species on the existing continuum between generalists and specialists and to confirm the role of the existence of the preference-performance relationship in the specificity of host of these parasitoids. Indeed, the parasitoids *B. communis* and *B. koreanus*, specialists of aphids from the genus *Aphis*, have the lowest host specificity and a relationship between their preference and performance has been described (Desneux et al. 2009 a, b). On another hand, parasitoids *A. abdominalis* and *D. rapae* have not a positive relationship between their preference and performance and show the highest indices of host specificity. Finally, parasitoids *L. testaceipes* and *A. ervi* are specialists of aphids belonging to the Aphidini and Macrosiphini tribe, respectively, have a specificity index in the medium range and show a relationship between their preference and performance.

Our data are therefore consistent with a general proposition that an insect oviposition strategy can be a complex compromise between many, sometimes contradictory, factors, including host range, clutch size, quality of host, difficulty of finding hosts of sufficient quality, chances to find even better hosts, risks of predation on the offspring, mobility of larvae and host-finding ability, microclimate, etc. Thus, although the main objective of the female may be to choose an

oviposition site that maximizes the survival and performance of the offspring, the outcome does not necessarily correspond to a perfect match between the host preference of the female and the larval performance. It will be now interesting to extend this work since we need a better knowledge of the taxon-specific idiosyncrasies to be able to make better generalizations as well as to sharpen our specific hypotheses.

However, the PPH and the host specificity index in parasitoids may provide useful cues for classifying parasitoids in terms of degree of specialization. Therefore, these efficacy criteria for biological control agents (e.g. wasps) could suggest whether parasitoids have good host selection capacity (Mills and Wajnberg 2008). The use of the approach described in this work could also help to represent the specificity of choice of wasps, which is now mandatory to be tested in many national regulations before releasing a biological control agent (Sundh and Goettel 2012).



PART 2 - THE APHID IMMUNE SYSTEM

Innate immunity

All organisms are constantly confronted with potential pathogens belonging to several categories (viruses, bacteria, parasites, fungi...). These enemies can invade the insect by different routes: respiratory tract, digestive tract, hemolymph by cuticular wounds such as the hole made by the ovipositor in the case of parasitoid (Fig. 12). When the physical barriers are crossed, the immune system tries to contain the invasion. The immune system is traditionally divided into two domains: "innate" and "adaptive" acquired immunity (Yatim and Lakkis 2015). Since adaptive immunity does not exist in insects, only innate immunity (whose constituents have remained constant over time (Sheehan et al. 2018)) makes it possible to defend against infection (Lemaitre and Hoffmann 2007; Kemp and Imler 2009; Hillyer 2016). This insect immune response is mediated by hemocytes, the fat body, the midgut, the salivary glands and other tissues.

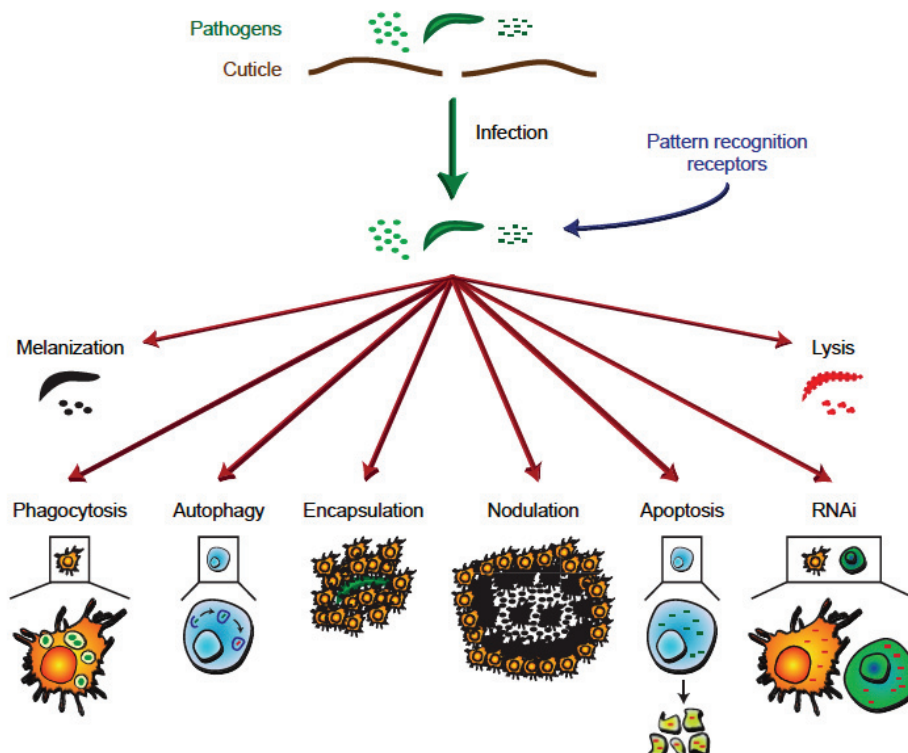


Fig. 12. Immune effector mechanisms of insects. Disruption of the epidermis and cuticle initiates an immediate response and wound sites serve as entry ports for pathogens. Wound signals attract hemocytes and activate PRRs and damage receptors. Pathogens can be killed via different mechanisms: phagocytosis, melanization, cellular encapsulation, nodulation, lysis, RNA interference, autophagy, and apoptosis. (see Hillyer 2016 further details).

The humoral component of the response includes the production of effector molecules, reactive oxygen species (ROS, NOS) and enzyme cascades that regulate lysis, coagulation and melanization processes. Hemocytes are essential players in the cellular response. They are also involved in phagocytosis, nodulation and encapsulation, the immune response to intrusion of a large foreign body (such as a parasitoid egg, too large to be phagocytized). Encapsulation involves hemocytes and humoral factors and it is evolutionarily conserved in insects. This is a visible proof that the immunity of the host responds to the aggressor. The success of the encapsulation reaction is reflected in the death of the parasitoid egg and in the survival of the insect host. Conversely, if the host larva is immunodeficient or the encapsulation response is inhibited, a parasitoid wasp will emerge. In insects, the fruit fly *Drosophila melanogaster* serves as a model for immunity (Lemaitre and Hoffmann 2007; Buchon et al. 2014; Rosales 2017), although studies on other Diptera (Rosales 2017; Kumar et al. 2018), Lepidoptera (Jiang et al. 2010; Rosales 2017), Coleoptera (Maire et al. 2018; Vigneron et al. 2019) and Hymenoptera (Evans et al. 2006), all holometabolous insects, are now available.

Curiously, the immunity of aphids has received little attention until recently. Aphids are expected to encounter few bacteria, because of their diet, and have been described primarily for their interactions with parasitoid wasps and some fungi. Aphids can be seen as species whose immune phenotype is mainly determined by their own genome or as meta-organisms whose immune defenses result from the combined effects of different genomes, including those of symbionts. To date, the protection mechanisms described are mainly those conferred by symbionts. Most information on the immunity of *A. pisum* are derived from genome sequencing data (International Aphid Genomics Consortium 2010). There is at least 18,000 genes predicted in *A. pisum* genome and it has been shown that in aphids whose genome has been sequenced that gene duplication has been pervasive throughout their evolution, including many parallel waves of recent, species-specific duplications (Tagu et al. 2010; Julca et al. 2019). A first annotation of the genes potentially involved in immunity was carried out by Gerardo et al. (2010). It highlights the absence of certain genes involved in the recognition of microorganisms, in certain signaling pathways and in the production of effector molecules (Table 8).

Table 8. Comparison of main immune genes from different species across selected insect orders. Coleoptera: T.mol, *Tenebrio molitor*; T.cas, *Tribolium castaneum* / Diptera: A.gam, *Anopheles gambiae*; D.mel, *Drosophila melanogaster* / Hemiptera: A.pis, *Acyrtosiphon pisum*; N.lug, *Nilaparvata lugens* / Hymenoptera: A.mel, *Apis mellifera*; N.vit, *Nasonia vitripennis* / Lepidoptera: B.mor, *Bombyx mori*; M.sex, *Manduca sexta* / Odonata: C.pue, *Coenagrion puella* / Phthiraptera: P.hum, *Pediculus humanus*. (from Viljakainen 2015).

Gene function	Pathway	Gene	Coleoptera		Diptera		Hemiptera		Hymenoptera			Lepidoptera		Odonata	Phthiraptera
			T. mol	T. cas	A. gam	D. mel	A. pis	N. lug	A. mel	N. vit	L. hum	B. mor	M. sex	C. pue	P. hum
Recognition		C-type lectin	12 ^a	16	25	34	5	9	10	31	12	21	4	?	9
		Dscam	?	1	1	1	1	9	1	?	1	1	1	1	1
		Eater	?	?	1	1	0	1	0	1	0	0	?	?	?
		GNBPs ^b	3	3	7	6	2	7	2	3	4	4	4	2	0
		PGRPs	6	7	11	13	0	2	4	12	6	12	10	4	1
Signaling	Toll	TEPs	3	4	13	10	2	?	4	3	3	3	2	1	3
		Spätzle	7	7	6	6	6	8	2	9	5	3	1	1	3
		Toll	2	9	10	9	6	6	5	9	6	14	1	3	6
		MyD88	1	1	1	1	1	2	1	1	1	1	1	1	1
		tube	1	1	1	1	1	1	1	1	1	1	1	?	1
		pelle	1	1	1	1	1	1	1	1	1	1	1	1	1
		cactus	1	1	1	1	1	1	3	1	2	1	1	1	1
		dorsal	1	1	1	2	2	1	2	1	1	1	1	1	1
		imd	1	1	1	1	0	1	1	1	1	1	1	1	0
		FADD	1	1	1	1	0	?	1	1	1	1	1	?	0
Signaling	Imd	Dredd	1	1	1	1	0	1	1	1	1	1	1	1	1
		IAP2	1	1	1	1	1	1	1	1	1	1	1	1	1
		TAK1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Tab2	1	1	1	1	1	1	1	1	1	1	1	1	1
		IKK β /ird5	2	1	1	1	1	1	1	1	1	1	1	1	1
		IKK γ /key	1	1	1	1	0	1	1	?	1	1	1	1	?
		Relish	2	1	1	1	0	1	2	6	1	1	2	1	1
		domeless	1	1	1	1	4	1	1	1	1	1	1	1	1
		hopscotch (JAK)	?	1	1	1	1	1	1	1	1	1	1	1	1
		Stat92E	1	1	2	1	2	1	1	1	1	1	1	1	1
Signaling	JNK	JNK/basket	3	1	1	1	1	?	1	1	1	1	1	1	
		hemipterous	1	1	1	1	1	?	1	1	1	1	1	1	
		Jra/Jun	?	1	1	1	1	?	1	1	1	1	1	1	
Effector		defensin	1	4	4	1	0	2	2	5	1	?	?	2	
		other AMPs	11	8	5	19	6	0	4	39	5	21	19	?	
		lysozyme	4	4	8	17	3	8	3	2	2	4	2	?	
		NOS	?	1	1	1	1	1	1	1	1	2	1	?	
Modulator		cSP	19	48	59	47	6	12	18	13	8	15	6	?	
		serpin	6	31	21	30	14	9	7	12	7	26	12	?	
Melanization		PPO	2	3	9	3	2	?	1	9	1	2	2	?	
RNA interference		Ago-2	?	1	1	1	?	?	1	1	1	1	1	1	
		Dcr-2	?	1	1	1	?	?	1	1	1	1	1	?	

Pathogen recognition

When a foreign body enters the hemocoel of an insect, this results in both the production of molecules with damage associated motifs (DAMP) and the presence of pathogen associated motifs (PAMPs, also known as microbe-associated molecular patterns or MAMPs). These products bind to receptors that recognize these patterns (PRRs, Pattern Recognition Receptors) and trigger subsequent effector mechanisms at the cellular level, such as phagocytosis and the production of antimicrobial peptides (AMPs) (Buchon et al. 2014; Rosales 2017). PRRs recognize the conserved motifs present in microbes but absent in insects, such as bacterial peptidoglycans and fungal beta-1,3 glucans.

PRRs are divided into a variety of conserved protein families such as peptidoglycan recognition proteins (PGRP), beta-1,3 glucan recognition proteins (also known as the Gram-negative binding proteins (GNBPs)), C-type lectins and leucine-rich repeat containing proteins (LRRs).

Many families of PRR proteins have expanded or contracted in different taxa, which is likely a consequence of the considerable differences in the ecology of insects (Table 8). For example, there are many members of the PGRP family in Diptera, Hymenoptera, Lepidoptera and Coleoptera analyzed to date but no PGRPs has been found in pea aphid (Gerardo et al. 2010) and in some of the other published aphid genomes (Gauthier et al. 2007).

In agreement, a subtractive hybridization (SSH) screening experiment identified 35 genes induced after septic injury in *A. pisum* (Altincicek et al. 2008). These genes encode proteins that are primarily involved in cell signaling, homeostasis and stress response, but no antibacterial peptide or PRRs (Pathogen Recognition Receptor) (Altincicek et al. 2008). The prick itself seems to induce a very weakly melanized coagulation reaction compared to the observations in other models but leads to an accelerated reproduction of aphids (see also further). Finally, although bacterial infection-induced lysozyme-like (muramidase) activity was detected in the hemolymph of aphids, no antibacterial activity has been detected against *E. coli* and *M. luteus*. These data, as well as more recent data from Gerardo et al (2010), suggest a weak antibacterial response in *A. pisum*, in line with the high sensitivity of aphids to bacterial infections (Altincicek et al. 2011). It was also suggested that this limited ability to mount a strong immune response to pathogenic bacteria was offset by an increase in fertility (Barribeau et al. 2010). Because of their prodigious reproductive capacity, a small increase in aphid fecundity can quickly multiply their number and may radically alter the competitive interactions at the clone level.

Immune pathways

Two cellular signaling pathways control inducible immune responses to bacteria and fungi in *D. melanogaster*: The Toll pathway and the immune deficiency (Imd) pathway (Fig. 13). The Toll pathway is active in the fat body and, together with the Imd pathway, controls the systemic production of antimicrobial peptides (AMPs). The Imd pathway is also active on the epithelial barrier surfaces, and it mediates the antimicrobial responses in association with enzymes producing reactive oxygen species (ROS), such as dual oxidase (Duox). These pathways are activated in response to the detection of microbial cell wall components. The Imd pathway is activated via the recognition of peptidoglycans of Gram-negative bacteria and certain Gram-positive bacteria by PGRP-LC and -LE and their binding to cell surface receptors. Toll is activated by PGRP-SA, GGBP1 and GGBP 3 which activate a proteolytic cascade that culminates in the cleavage of the cytokine Spätzle, which is mediated by the Spätzle protease processing enzyme (SPE). In addition, the Toll pathway can be activated through microbial

proteases or abnormal cell death that triggers activation of the Persephone protease. Activation of Toll and Imd ultimately leads to nuclear translocation of the nuclear factor- κ B (NF- κ B) transcription factor (Dif and Relish, respectively) leading to expression of AMP genes and other genes.

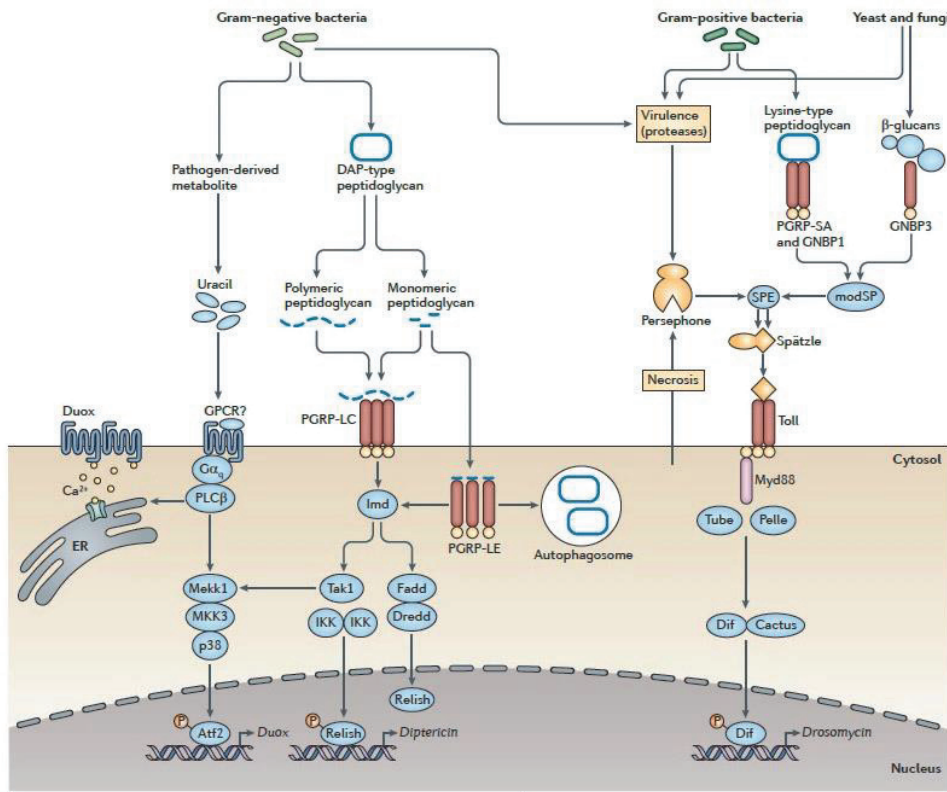


Fig. 13. Immune recognition of pathogens in *Drosophila melanogaster*. (from Buchon et al. 2014).

These signaling pathways are well conserved in a wide taxonomic range of insects, with the exception of *A. pisum* (Gerardo et al. 2010) (see also Table 8). Pea aphid lacks most microbial recognition genes such as the peptidoglycan receptor proteins (PGRPs) involved in the Imd and Toll pathways, as well as class C scavenger receptors and epidermal growth factor (EGF)-repeat-containing genes found in the receptors of phagocytosis and bacterial binding in other insects. Although the Toll and Janus kinase/signal transducer (JAK/STAT) pathways are almost complete, perhaps because they are also involved in the development (Belvin and Anderson 1996), many components of the Imd signaling pathway, are missing (*imd* itself, *Dredd*, *FADD*...).

Activation of these pathways leads to increased gene expression, including different types of AMPs. AMPs are small peptides characterized by a positive overall positive charge (cationic), hydrophobicity and amphipathicity. They can be classified into families on the basis of their

tertiary structure and/or amino acid sequence, in particular the conserved cysteine motif (Wu et al. 2018). AMPs in insects show lineage specificity both in copy number in a gene family and in the presence/absence of a complete gene family. For example, the AMP family of Coleoptericin is present only in the order Coleoptera (Bulet et al. 1991) and the *Drosomycin* family in certain *Drosophila* (Bulet 1999). Due to the specificity of the lineage and the potential for sequence divergence, the identification of novel AMPs in a sequenced genome is challenging. Pea aphids lack many antimicrobial peptides (AMPs) of gene families conserved in other insects, such as defensins found in almost all insect orders studied thus far (Table 8). So far, only genes encoding thaumatin-like peptides that exhibit antifungal activity in various plant species (Petre et al. 2011) have been identified in the pea aphid genome but, to our knowledge, their role in the protection of aphids is not demonstrated.

Hemocytes and Phagocytosis

In invertebrates, blood cells are commonly called hemocytes. It is possible to distinguish different types of hemocytes from most of the invertebrate species studied so far (Brehélin et al. 1978; Lavine and Strand 2002). Overall, four main categories of hemocytes are classically distinguished: prohemocytes, granulocytes, plasmatocytes and oenocytoids. Prohemocytes are small, round cells with a large nucleus surrounded by a relatively thin cytoplasm. Plasmatocytes are always larger than prohemocytes and their cytoplasm is considerably more prominent, the RER and the Golgi complexes are well developed, indicating a differentiated cell. Plasmatocytes are phagocytes among circulating hemocytes, although they are not the main hemocytes capable of phagocytosis. Granulocytes are large polymorphic cells that contain uniformly dense round or ovoid granules. In most species investigated, granulocytes do not participate in phagocytosis of foreign bodies or histolytic debris. Oenocytoids are very large cells are remarkably poor in cytoplasmic organelles. Their RER appears as dispersed vesicles and the scarce mitochondrial profiles often have an annular shape. Other cell types described, such as thrombocytes and podocytes, have been observed in only a very small number of species (Brehélin et al. 1978). However, new methods can increase the number of type and subtypes of cells and describe their complex lineage (Márkus et al. 2015; Ghosh et al. 2015). In *Drosophila*, the terminology used to describe the hemocytes is different: prohemocytes are cells that can differentiate in the other three types of hemocytes, phagocytic cells are called plasmatocytes, large cells dedicated to encapsulation are called plasmatocytes in most insect species but lamellocytes in *Drosophila*. Finally, oenocytoids are called crystal cells. These hemocytes can be identified by a combination of morphological, antigenic and functional characteristics.

While hematopoiesis is well described in *Drosophila* and several other invertebrates and the presence of hematopoietic organs demonstrated (Meister and Lagueux 2003; Crozatier and Meister 2007; Grigorian and Hartenstein 2013; Braquart-Varnier 2015; Hillyer 2016), aphid hemocytes have been described in detail only recently and their origin has not yet been defined. The pea aphid contains between 1440 and 1800 hemocytes/ μl of hemolymph (Laughton et al. 2011; Schmitz et al. 2012). In comparison, *Drosophila* L3 larvae contain about 5000 hemocytes/ μl (Labrosse et al. 2005). A study from the laboratory (Schmitz et al. 2012) established at least two classes of hemocytes based on their adhesion properties: the first class was composed of three types of adherent hemocytes named prohemocytes (although with low adhesion properties), plasmatocytes and granulocytes and the second class consisted of two types of large non-adherent cells, oenocytoids and wax cells (Fig. 14). These cells were classified according to the criteria and morphological aspect defined previously, as well as functional aspects. Prohemocytes account for about 5% of circulating hemocytes, the majority being granulocytes and plasmatocytes. These two types of cell are phagocytes capable of engulfing latex beads and *E. coli*, while oenocytoids are very fragile cells releasing very quickly their contents which form a large extracellular trap such as neutrophils involved in netosis (de Bont et al. 2019). The wax cells are similar to the cells described at the base of the cornicles and are certainly involved in behavioral defense by spreading wax and alarm pheromone (Edwards 1966; Callow et al. 1973).

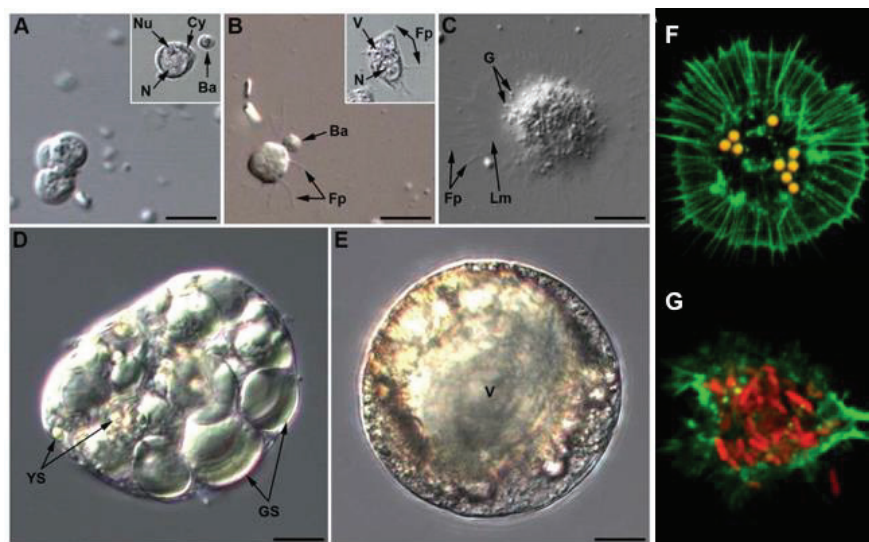


Fig. 14. Light microscopy pictures of *Acyrthosiphon pisum* hemocytes (LL01 clone). (A) Three prohemocytes in cluster. Inset: showing the large central nucleus (N) and the nucleolus (Nu). Ba: *B. aphidicola*. (B) Plasmatocyte starts to adhere, with filopodia (Fp) extension. Inset: large cytoplasmic vacuolar (V) formation. (C) Adherent granulocyte containing cytoplasmic granules (G) and filopodia (Fp) extending from a lamellipodium (Lm). (D) Spherulocyte with its large colored globular inclusions, small yellow spherules (YS) and large green spherules (GS). (E) Wax cell showing a large central vacuole (V) and colored globular inclusions that differ from those of spherulocytes. Same magnification for all micrographs; scale bar: 10 μm . (F and G) Confocal images showing *in vivo* phagocytosis by granulocytes of yellow fluorescent latex beads (F) and red fluorescent *E. coli* 24 h post-injection in LL01 aphids. (From Schmitz et al. 2012).

More surprising was the observation of adherent hemocytes of pea aphid lines whose hemolymph contains a large number of secondary symbionts. Different methods have revealed the presence of circulating symbionts inside the hemocytes and even the property of phagocytosing the primary symbiont when in contact. Using FISH, it was shown that a high proportion of plasmatocytes and granulocytes have their cytoplasm full of bacteria (data shown for *Ri*), Fig. 15). The occurrence of phagocytosis was further confirmed by transmission electron microscopy in granulocytes of different lines infected with different symbionts. *Hamiltonella defensa* (*Hd*) symbionts were found in phagosomes but no phagolysosomes-like and *Regiella insecticola* (*Ri*) in phagolysosome-like structures. In both cases, the bacterium showed no visible sign of degradation. *Serratia symbiotica* (*Ss*) was only found in some concentric multi-membrane phagosomes suggesting complete degradation of the bacterium. This demonstrated that symbionts circulating in the hemolymph could be phagocytosed by hemocytes. It was suggested that *Ss*, considered a low pathogenic symbiont, be eliminated, while *Ri* and *Hd*, carriers of active T3SS and producing putative virulence factors, may be able to survive/hide inside the hemocytes, by blocking or slowing down the phagocytic process (Schmitz et al. 2012).

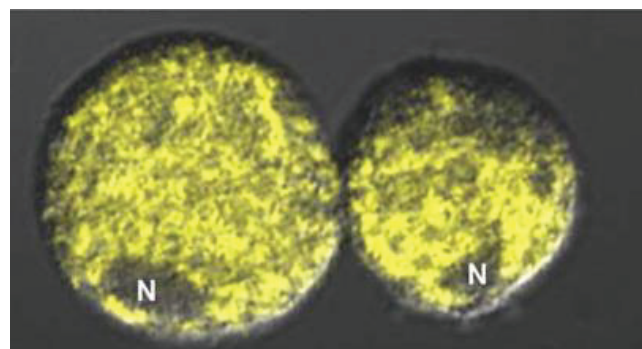


Fig. 15. *In vivo* phagocytosis of symbionts by hemocytes. Specific detection of *Regiella insecticola* by FISH (yellow dots, false color; DIC and confocal micrographs overlay). Nucleus (N) location is detectable by the absence of yellow coloration. (From Schmitz et al. 2012).

Symbiont effects on cellular immunity

Studies on the effects of symbionts on host cell immunity revealed contrasting effects suggesting a possible variation in host-symbiont genotypic interactions. The elimination of *Wigglesworthia glossinidia*, the obligate symbiont of *Glossina morsitans*, during larval development compromises the immune response of the tsetse fly to a microbial challenge (Pais et al. 2008). The expression of genes encoding AMPs is decreased, and the number of hemocytes and hemocyte-mediated processes is reduced. *Wigglesworthia* is therefore required

during development for a functional immune system in adult flies. On the contrary, the immune defense of *Sitophilus oryzae* weevil is lowered in larvae harboring the obligate Gram-negative γ -proteobacterium *Sodalis pierantonius*, compared to aposymbiotic ones, suggesting a down-regulation of immunity when in symbiosis (Vigneron et al. 2012). At last, the facultative presence of *Wolbachia* in the crustacean *Armadillidium vulgare* causes a change in the proportion of hemocyte types and a reduction in their number in aged specimens (Sicard et al. 2010).

In pea aphid, the presence of some secondary symbionts can affect the cellular immunity of aphids (Schmitz et al. 2012; Laughton et al. 2016). In their study, Schmitz et al. (2012) found a clear difference in the number of hemocytes between lines devoid of secondary symbionts and similar lines (genotype YR2) with either a natural *Ri* symbiont or artificially infected with *Hd* or *Ss* (Fig. 16). Although the origin of this difference in number was not identified, only the presence of the two more pathogenic symbionts, *Hd* and *Ri*, had an effect, not that of *Ss*.

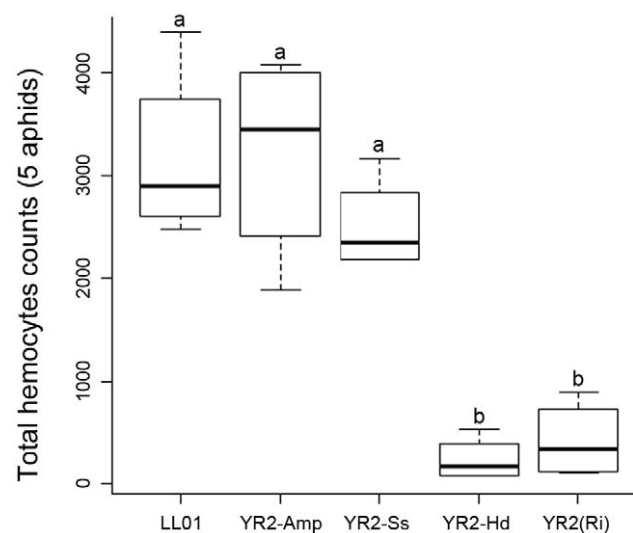


Fig. 16. Total hemocyte counts (THC) from different symbiotic lines. The total number of adherent hemocytes from the LL01 (naturally devoid of secondary symbiont) and YR2 lines either naturally infected with *Ri* (YR2(*Ri*)), devoid of symbiont due to antibiotic treatment (YR2-Amp), or artificially infected with *Hd* or *Ss* (microinjection in YR2-Amp). The same letter means that are no significantly difference. (Schmitz et al. 2012).

Melanization, nodulation and encapsulation

The melanization pathway

Melanization covers all complex enzymatic and spontaneous reactions that lead to the formation of different types of compounds depending on the conditions: eumelanin (brown-black), a highly heterogeneous copolymer consisting of DHI (DiHydroIndole) and DHICA (DiHydroxyIndole Carboxylic Acid) units in reduced or oxidized form, and pheomelanin

mainly composed of benzothiazine derivatives containing Sulphur (Fig. 17) (Vavricka et al. 2010). It is an important reaction in most multicellular organisms, both animals and plants. In vertebrates, melanin provides pigmentation and protection and is important for the development of the central nervous system and eyes, among others. In most invertebrates, the production of melanin (eumelanin and pheomelanin) is vital for many physiological processes, including sclerotization of the cuticle (tanning) post-ecdysis, hemostasis and wound repair, pigmentation of the wings and eyes, as well as gut homeostasis.

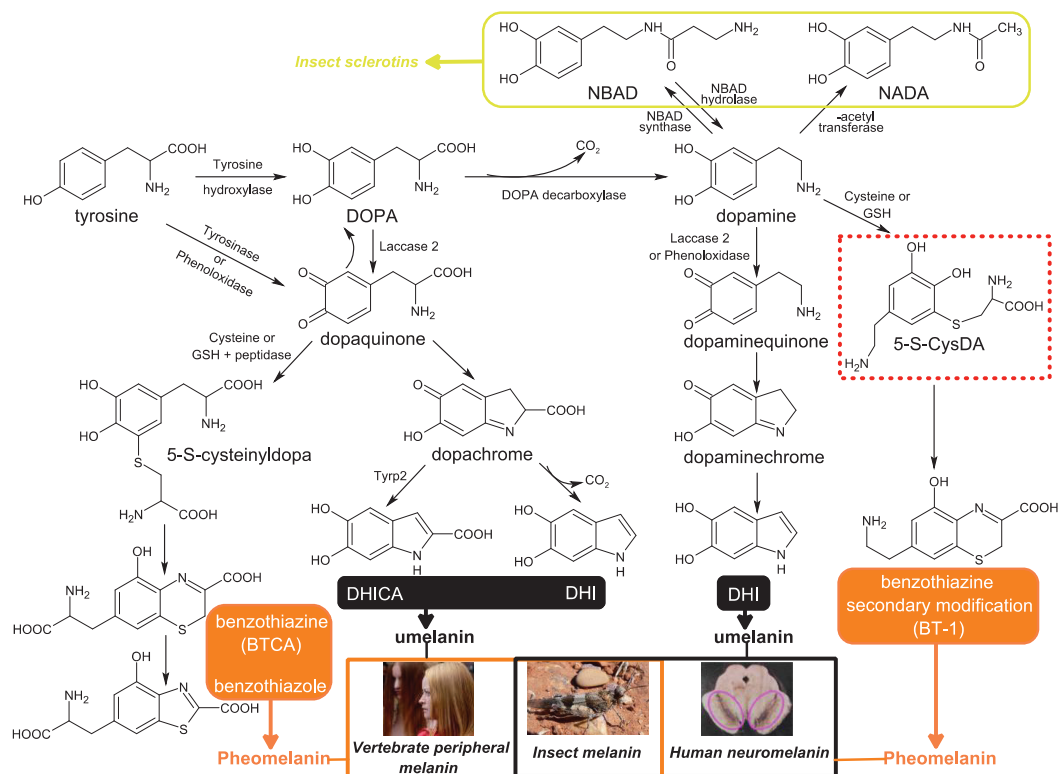


Fig. 17. Schematic representation of the biochemical pathway leading to synthesis of vertebrate and insect melanins from DOPA and from DA and to the synthesis of insect sclerotins from dopamine. Melanin formation in insects and vertebrates, including the DA pathway, coinciding with the formation of the pheomelanin moiety of human neuromelanin. The neurotoxic intermediate cys-DA is marked in red. Arrows without associated enzymes represent non-enzymatic reactions. (from Galván et al. 2015).

The phenoloxidase system

The pro-phenoloxidase system (PPO) is active in many invertebrates and, regardless of their phylogenetic position, animals produce melanin in both body fluids and/or in cells. In insects, the PPO system and its activation have been extensively studied in Lepidoptera, such as *Bombyx mori* and *Manduca sexta*, and Diptera, such as *D. melanogaster* and different mosquitoes (Whitten and Coates 2017). In insects, the number of PPO genes varies from 1 (e.g. in *Apis*

melifera) to 10 in the mosquito *Aedes aegypti* (Whitten and Coates 2017). In *Drosophila*, three genes encode three different PPOs (PPO1, PPO2, PPO3). These enzymes are present in specialized blood cells (crystal cells and lamellocytes) and in a circulating form for PPO2 and they are released from cells into the hemolymph or wounding place upon stimulation. PPO1 and PPO2 play an essential role in survival after infection by Gram-positive bacteria and fungi (Nappi 2009; Eleftherianos and Revenis 2011; Binggeli et al. 2014; Nakhleh et al. 2017; Dudzic et al. 2019). Prophenoloxidases are inactive proenzymatic forms. Activation of PPO to synthesize melanin is triggered with the recognition of microbial cell surface molecules, such as peptidoglycans or lipopolysaccharides (LPS) of bacteria and β -1,3-glucans of fungi, by pattern recognition proteins (Fig. 18) (Nappi 2009; Eleftherianos and Revenis 2011; Nakhleh et al. 2017; Dudzic et al. 2019). This system includes in many insects a cascade of serine protease-serine protease inhibitor (Serpine) which ultimately cleaves the prophenoloxidase into active phenoloxidase (PO). Interestingly, although *D. melanogaster* PPO1 and PPO2 require proteolytic cleavage to be activated, it is believed that PPO3 is produced in an active form, although a putative cleavage site is present (Chen et al. 2012).

The PO, once active, catalyzes the first reaction chain by forming indole groups from tyrosine, which are then polymerized to melanin. The enzymatic and spontaneous reactions produce in turn a set of intermediate products, such as quinones, diphenols, superoxide, hydrogen peroxide and reactive nitrogen intermediates, essential for the destruction of invaders. Melanization is a widespread immune response essential for isolating and inactivating any type of non-self at the site of injury and/or on the surface of foreign invaders. The biosynthesis of melanin and pigment precursors is essential in invertebrates in which invaders such as parasites or fungi are rapidly isolated and sequestered in capsules made of pigment and circulating cells. In addition, PO cascade also appears to function in other immune pathways, such as antimicrobial peptides (AMPs) synthesis (Lemaitre et al. 1995; Matskevich et al. 2010), suggesting a potential crosstalk between melanization and other immune pathways to coordinate the immune response in resisting invaders. Some evidence suggests that monomeric PO may not be the terminal step in the PPO activation cascade. In fact, POs were found to interact with various macromolecules such as serine protease homologues (SPHs) in the Lepidoptera *M. sexta* (Gupta et al. 2005) and *B. mori* (Clark and Strand 2013), and membrane phospholipids (Bidla et al. 2009) in *D. melanogaster*, thereby forming “activation complexes”.

A. pisum has two annotated prophenoloxidases in its genome (ProPO1 and ProPO2) that group with *D. melanogaster* PPO2 and PPO3, respectively. Both enzymes mRNA are expressed and the phenoloxidase activity can be measured in the aphid (Gerardo et al. 2010).

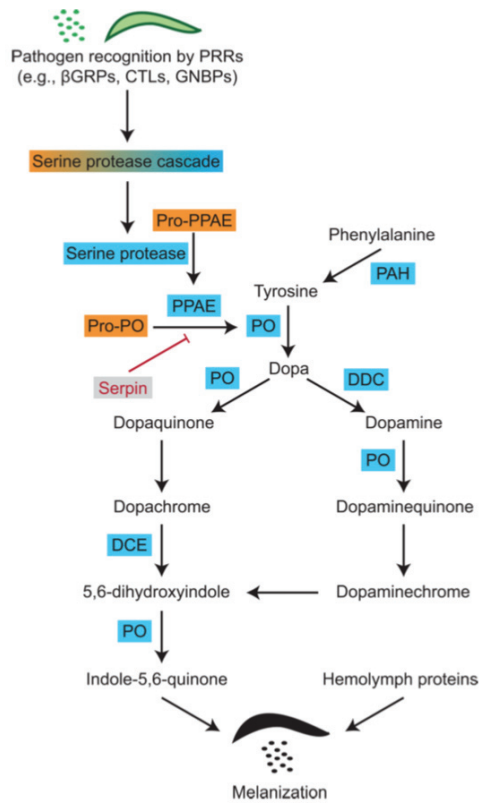


Fig. 18. Biochemical pathway of phenoloxidase-based melanization in insects. For a description of the pathway, see in the text. Abbreviations: PRR, pattern recognition receptor; β GRP, β -1,3 glucan recognition protein; CTL, C-type lectin; GGBP, Gram (-) binding protein; PPAE, prophenoloxidase activating enzyme; PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme. (From Hillyer 2016).

Coagulation/clotting

After an injury, coagulation of the hemolymph is necessary to maintain hemostasis and prevent the entry and internal dissemination of pathogens. It takes place in two phases. The wound is initially covered with a soft clot formed by the aggregation of transglutaminase-crosslinked clot proteins, a component of clotting cascades conserved during evolution (Dushay 2009). Then the clot hardens through PO-dependent cross-linking (Li et al. 2002). When the wound is infected, the pathogens are usually contained in smaller clot aggregates (Patterson et al. 2013; Nakhleh et al. 2017). Coagulation requires humoral components provided by the fat body and cellular proteins, which are produced by the hemocytes. Lipophorin, hexamerins and phenoloxidase are among the main humoral coagulation factors in *Drosophila*. In this species, plasmatocytes and crystal cells contribute to clotting by producing hemolectin as well as complement factor and phenoloxidase (Lee and Miura 2014). Coagulation occurs in aphids, although the reaction seems limited compared with other insect species (Altincicek et al. 2008). Piercing *A. pisum* with a sterile or bacteria-contaminated needle resulted in relatively low coagulation and restricted melanization (Fig. 19) and, even 24 h after, the wounds were not sufficiently sealed since the hemolymph was still leaking out.

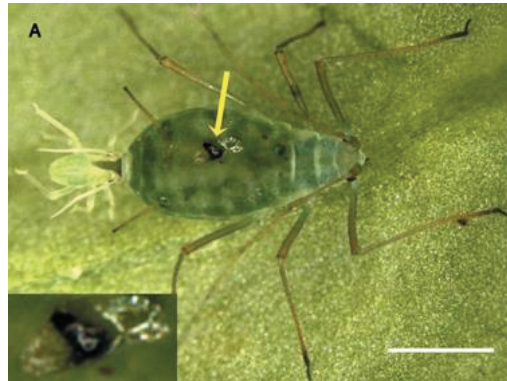


Fig. 19. Picture of a wounded pea aphid giving birth to offspring. At the wounding site (indicated by an arrow and shown magnified as insert) there was a low hemolymph coagulation reaction and partial melanization of the exposed hemolymph. Note that the melanization of exposed hemolymph is complete. Scale bar = 1 mm. (from Altincicek et al. 2008).

Nodulation and encapsulation

Nodulation and encapsulation are innate immune responses more effective against a large number of pathogens or metazoan parasites in insects (Rizki 1968), leading to sequestration of the invader with cells and biopolymers of melanin and sclerotin, as well as proteins (Tokura et al. 2014). The formation of nodules is a mixture, to a variable extent, of phagocytosis, melanization and encapsulation (Bedick et al. 2001). At one extreme, some blood cells that have engulfed foreign particles, mainly pathogens (bacteria and fungi), adhere to form a small clump without a constant structure. At the other extreme, a large nodule has the lamellated structure forming a capsule embedding the invaders; indeed, it is a capsule of which the layer does not join on a continuous surface but merges into an agglomeration of degenerating blood cells, foreign particles, and melanized debris. Nodulation mechanisms are poorly understood compared to other immune responses. It has been shown that eicosanoids mediate nodulation in many insect species (Bedick et al. 2001) and that specific proteins such as noduler, a protein that may bind extracellular matrix proteins, are enriched in nodules (Gandhe et al. 2007).

Encapsulation is in general more complex than nodulation. For example, in *Drosophila*, it includes the coordinated action of multiple hemocyte types binding to larger invaders, like protozoans, nematodes and parasitoids (eggs and larvae), that cannot be phagocytized by a single cell (Fig. 20) (Vass and Nappi 2001; Vlisidou and Wood 2015; Kim-Jo et al. 2019). The formation of the cellular capsule results from a first layer of plasmatocytes followed by a successive accumulation of layers of specific cells whose presence in circulation is induced by parasitism, the lamellocytes (Nappi et al. 1973; Rizki and Rizki 1992). The hemocytes establish a junction resulting in continuous coverage on the target surface and the inner layers undergo partial disruption leading to melanization by activation of the proPO cascade. Reactive oxygen

(ROS) and nitrogen (RNS) species are emitted during melanogenesis and targeted against the invader (Nappi et al. 1995; Carton and Nappi 1997; Nappi and Ottaviani 2000). As a result, the intruder is not only isolated in the capsule, but also destroyed.

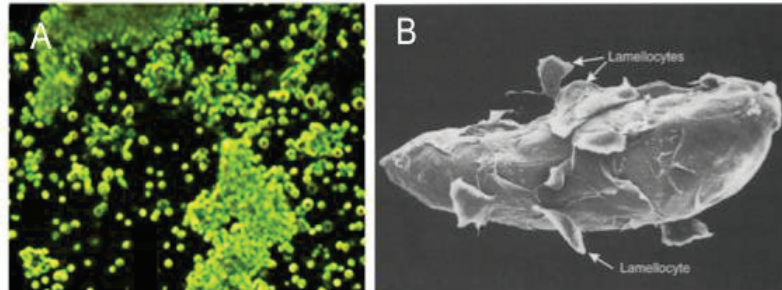


Fig. 20. Nodulation and encapsulation. (A) Micro aggregation reactions to bacterial infection in larvae of the diamond backmoth, *Plutella xylostella*, leading to nodule formation. (B) Scanning electron micrograph of an encapsulated egg of the parasitoid *Leptopilina boulardi* in the host *Drosophila melanogaster* approximately 20 hrs after infection. Lamellocytes flatten at the contact of the egg and form several successive layers. (from Stanley 2011)

Although encapsulation of parasitoids eggs has seldom been reported in aphids, it has been shown to involve granule-containing cells (Ankersmit et al. 1981; Carver and Woolcock 1985) that probably correspond to granulocytes. Two separate studies investigated the encapsulation response of pea aphid to a foreign object. Laughton et al. (2011) observed that pea aphids could rapidly form a melanic deposit around Sephadex beads injected into the hemocoel (Fig. 21). They also observed that some individuals (about 10%) did not melanized any beads during the experimental time. When melanization occurs, beads closer to the site of injection tend to be more strongly melanized than beads recovered elsewhere in the haemocoel, suggesting that melanization of the beads may be partially influenced by the proximity to the damaged cuticle of the aphid.

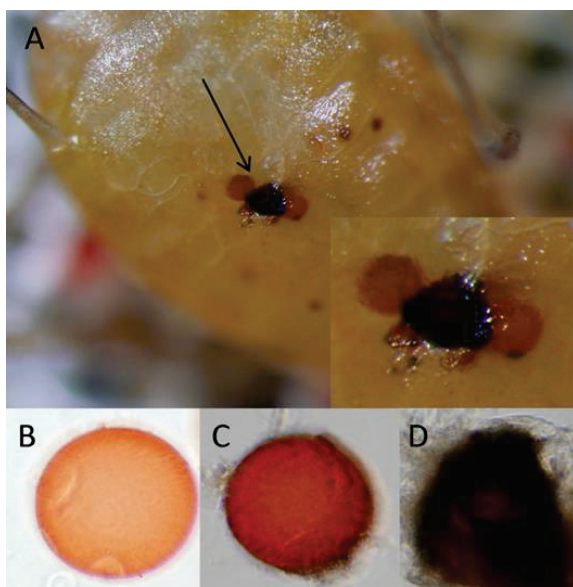


Fig. 21. Melanization of Sephadex beads injected into aphids. (A) Low magnification image showing three melanized beads in the aphid haemocoel (arrow). A higher magnification shows the variation in intensity of melanization among beads. (B) Control bead stained with Congo Red. (C) A representative bead that was weakly melanized. (D) Strongly melanized bead with maybe some visible cells around. (from Laughton et al. 2011).

Laughton et al. (2011) suggested that pea aphids do not form cellular capsules because: i) they have a low hemocyte count (about 500-600 hemocytes/aphids) unlike other hemimetabolous insects forming cellular capsules (for example, the hemolymph of the American cockroach, *Periplaneta americana*, contains 80,000 hemocytes/ μ l (Wheeler 1963)), ii) they lack a type of hemocyte specialized in capsule formation as found in different insect species.

Schmitz et al. (2012) inserted various inert objects into an aphid hemocoel to test the adhesion properties of hemocytes *in vivo*. The adhesion of some hemocytes was already observed 24h post-insertion, and their number increased with time. One week after insertion, the objects were covered with adherent hemocytes forming a more or less extensive cell monolayer, but which never happened to be complete (Fig. 22). On clear and transparent objects, dark/brown melanin deposits were observed. Most of the inserted hemocyte-covered objects were found attached to aphid tissues such as the fat body, trachea or digestive tract. At the ultrastructural level, the cell monolayer was composed of both plasmatocytes and granulocytes without apparent organization. Interestingly, an electron dense layer was always observed by TEM at the interface between the hemocytes and the surface of the inserted objects, even in areas devoid of hemocytes.

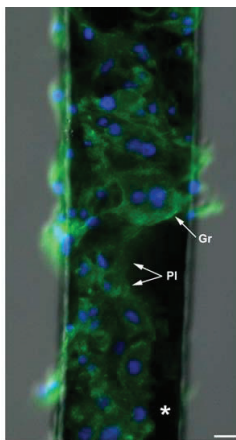


Fig. 22. Observations of the adhesion of hemocytes on a foreign object. Seven days after insertion of a brush horsehair (asterisk) in the hemocoel of an aphid, both plasmatocytes (Pl) and granulocytes (Gr) are observed (F-actin (green) and nuclei (blue) staining). The melanization could not be observed due to the dark color of the hair. Scale bar: 10 μ m. (From Schmitz et al. 2012).

Thus, in *A. pisum*, granulocytes, but also plasmatocytes, can adhere *in vivo* to foreign objects in a manner quite similar to the hemocytes of most insect species. However, the encapsulation reaction was never complete, possibly because of the low number of recruited hemocytes and/or a low recruitment rate. A melanin-like was also observed on the surface of foreign objects with possibly many cells undergoing lysis. In *A. pisum*, PO-positive granulocyte-type cells, as well as circulating granulocytes containing melanized latex beads in the hemolymph were observed (Laughton et al. 2011), suggesting that these cells could participate in melanization. It therefore seems that aphids can mount an encapsulation response but slowly and surely inefficiently in case of parasitism, the parasitoids developing rapidly and killing the host in a few days.

Publication 3. Lower amounts of phenoloxidases transcripts and proteins and modulated immune response in pea aphids harboring *Regiella insecticola* or *Hamiltonella defensa*.

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In prep

Lower amounts of phenoloxidases transcripts and proteins and modulated immune response in pea aphids harboring *Regiella insecticola* or *Hamiltonella defensa*.

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Abstract

The pea aphid, *Acyrtosiphon pisum*, harbors the obligate symbiont, *Buchnera aphidicola*, which is necessary for its metabolism. It can also host one or more of nine facultative symbionts (FS), which provide various extended phenotypes to the host, including adaptation to the host plant and resistance to heat or biotic agents (fungi, bacteria, parasitoid wasps). Here, we searched whether and how the presence of some FS could affect one of the components of insect innate immunity, the phenoloxidase cascade, under normal and stressed conditions. For this, we used *A. pisum* clones of different genetic background and without FS (LL01, YR2-Amp, T3-8V1-Amp) and natural or artificial YR2 or T3-8V1 lines hosting one of the following FS: *Regiella insecticola*, *Hamiltonella defensa* or *Serratia symbiotica*. Proteomic analysis of the LL01 hemolymph and molecular analyses indicate that the two phenoloxidases, PO2 and PO2-X1 are expressed. They seem mainly secreted as circulating enzymes in the hemolymph and do not need proteolytic cleavage to be activated. We observed a genotype effect on the expression of PO genes and the amount and activity of PO proteins in the total hemolymph (T3-8V1-Amp > LL01 = YR2-Amp). Also, the presence in YR2 and T3-8V1 of *H. defensa* or *R. insecticola*, but not *S. symbiotica*, caused a sharp decrease in all these parameters by interfering with the levels of both transcription and translation of PO. Microinjection of different types of stressors (yeast, *E. coli*, latex beads) in YR2 lines mainly affected the survival rate of aphids. In most cases, it also decreased the expression of PO genes after 24h, whereas the amount and activity of the proteins varied differently depending on the FS and the stressor, regardless of genes expression.

Key words

Pea aphid, *Acyrtosiphon pisum*, Phenoloxidases, Hemolymph, Facultative symbionts, *Regiella insecticola*, *Hamiltonella defensa*, *Serratia symbiotica*, stressors.

Introduction

The pea aphid *Acyrtosiphon pisum* is an agricultural pest used as a model for studying host-symbionts interactions. Most aphids live a long-lasting obligatory symbiosis since 150 million years with an alpha-proteobacterium, *Buchnera aphidicola*, which is housed in specific cells called bacteriocytes (Buchner, 1965; Douglas, 1989; Fukatsu, 1994; Baumann et al. 1997; Braendle et al. 2003). This symbiont fulfills the nutritional needs of aphids by supplementing their deficient sap diet with essential amino acids. It is therefore essential for the survival and reproduction (Wilson et al. 2010; Colella et al. 2018) of the host. In addition to *B. aphidicola*, aphids can also host one or more of nine different facultative symbionts (FS) that are mainly transmitted vertically and coexist with *Buchnera* (Guo et al. 2017; Guyomar et al. 2018). These FS have been found in secondary bacteriocytes, free in the hemolymph and more recently in the hemocytes (Tsuchida et al. 2005; Schmitz et al, 2012). *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica* are the most common FS in *A. pisum* (Moran et al., 2005; Ferrari et al., 2012; Gauthier et al. 2015). These facultative symbionts are maternally transmitted and provide various extended phenotypes to aphids, ranging from adaptation to the host plant to resistance to biotic (pathogens, parasitoids) or abiotic (heat) stresses (Montllor et al., 2002; Oliver et al., 2003; Scarborough et al., 2005; Oliver et al., 2010; Łukasik et al., 2013). Bearing FS can also induce fitness costs to aphids (Simon et al. 2011; Martinez et al. 2017; Cayinetano et al. 2014) and can affect their behavior (Dion et al. 2011).

Insects have an innate immune system (Lemaitre and Hoffmann 2007, Hillyer 2016, Keehnen et al., 2017) that allow them to survive injuries and defend themselves from pathogens and parasites (Lemaitre and Hoffmann 2007; Hillyer 2016; Keehnen et al. 2017). An interesting question is therefore how bacterial symbionts can persist despite these immune defenses? Insect innate immunity involves different humoral and cellular defense mechanisms mobilized differentially according to insect species and types of aggression. In most species, humoral defenses against pathogens rely on pathogen recognition receptors (such as Peptidoglycan recognition proteins (PGRPs)) and the production of antimicrobial peptides (AMPs) and enzymes such as lysozymes and phenoloxidases (POs) that are then released in the hemolymph. The annotation of the pea aphid genome has shown the presence of many classical genes for insect immunity (e.g. Toll and JAK / STAT signaling pathways, phenoloxidases (PO)), but also the absence of some pivotal genes for the response to Gram-negative bacteria such as PGRPs and genes of the Imd signaling pathway (including the *imd* gene itself). Moreover, none of the genes encoding AMPs conventionally found in holometabolous insects could be identified, the only AMP genes found in the genome encoding thaumatin-like antifungal peptides (The

International Aphid Genomics Consortium, 2010, Liu et al. 2010). Such deficiency in anti-bacterial response to Gram-negative bacteria led to the hypothesis of an adaptive change of the immune system of the aphid associated with selection for protection of the bacterial symbionts (Gerardo et al., 2010; Burke and Moran 2011). For instance, the maintenance of the obligate symbiont associated with *Sitophilus zeamais* weevil (*Sodalis pierantonius*) in a specific organ, the bacteriome, is under the control of the strong expression in this organ of Coleopteracin A, an AMP that hampers bacterial cells division during replication, leading to the production of long bacterial filaments that cannot leave the bacteriome to enter the hemocoel (Login et al., 2011; Vigneron et al., 2012). In addition, bacteriome-specific cytosolic and transmembrane isoforms of *pgrp-lb* are permanently produced and specifically cleave the tracheal cytotoxin (TCT), a peptidoglycan monomer released by endosymbionts. Silencing this gene results in TCT escape from the bacteriome to other tissues, where it chronically activates the host systemic immunity (Maire et al. 2019). One drawback of this weakened immunity in aphids is that pathogen challenges or wounding hardly induce significant changes in the expression of known immunity genes (Altincicek et al., 2008; Gerardo et al., 2010). Pea aphids have limited ability to close cutaneous wounds by melanization and coagulation of the hemolymph (Altincicek et al., 2008) and the encapsulation response is a slow process may be due to a low number of hemocytes (Laughton et al., 2011; Schmitz et al., 2012). Besides, it seems ineffective against a parasitoid egg of the wasp *Aphidius ervi* since no immune cell seems to be able to adhere to it, and it develops rapidly after oviposition (Pennacchio et al. 1999; Oliver et al., 2005). However, Xu et al. (2019) showed that inhibition of PO expression led to an increase in number of bacterial cells and fungal spores in the aphid body and higher mortality after pathogenic infections, suggesting its potential role in the defense against these pathogens. It has also been proposed that the presence of facultative symbionts could shape the immune response of the host (Gross et al., 2009; Welchman et al., 2009). Indeed, the presence of certain facultative symbionts seems to negatively affect the immune components of the pea aphid i.e. the number of both types of adherent hemocytes, granulocytes and plasmatocytes (both having phagocytic and encapsulation properties) and the activity of phenoloxidase in the hemolymph (Schmitz et al., 2012; Laughton et al., 2016).

Here, based on previous laboratory results, we focused on the effect of the genetic background of the host and the presence of various facultative symbionts on the phenoloxidase system, by considering the different levels possibly affected. POs are important enzymes involved in the melanization of insects during cuticle sclerotization, wound healing, defense responses and the encapsulation process (Cerenius et al., 2008; Nappi, 2010; Eleftherianos and Revenis, 2011;

González-Santoyo and Córdoba-Aguilar 2011; Dudzic et al. 2015). The genome of *A. pisum* contains two PO genes named PO2 (ACYPI001367, previously ProPO1) and PO2 variant X1 (ACYPI004484, previously ProPO2), both being expressed (Gerardo et al., 2010; Xu et al. 2019). Here, we analyzed in detail the expression of the two PO genes by qPCR, the amount of the PO proteins in the hemolymph by Western blot and the PO activity via enzymatic conversion to L-DOPA. Different clones were used to test the effect of aphid genetic background, and lines of the same clone containing a single facultative symbiont to compare the effects of symbionts with each other. This also allowed disentangling the effects of the aphid genetic background from the effect of the FS.

We confirmed that both PO genes are expressed and that their product present in a free circulating form in the hemolymph. We found a high correlation between the expression of PO genes, the amounts of PO proteins and the hemolymph activity of PO, all being dependent on the host genetic background and strongly influenced by the presence of the facultative symbionts *H. defensa* and *R. insecticola*. We also observed that the effects of stressors on aphid's fitness traits correlated with the presence of some symbionts (and thereof the amount of aphid POs) but this correlation no longer existed after the stress. This study provides important insights to further identify the mechanism(s) by which the various facultative symbionts might modulate the host immune system and perhaps protect themselves from it, under normal or stressed conditions.

Materials and Methods

Aphids lines

Three distinct clones of *A. pisum*, LL01, YR2 and T3-8V1, were used. LL01 is a natural clone devoid of secondary symbionts (Rahbé et al. 1993) while YR2-Amp and T3-8V1-Amp (also devoid of secondary symbionts) were created by antibiotic treatment from clones from the field naturally harboring *R. insecticola* (Nyabuga et al. 2010; Simon et al. 2011). YR2-Ss, YR2-Ri(a), YR2-Hd and T3-8V1-Ss, T3-8V1-Hd were obtained by injecting respectively *S. symbiotica*, *R. insecticola* and *H. defensa*, into the YR2-Amp or the T3-8V1-Amp line (Nyabuga et al. 2010; Simon et al. 2011). The created lines that contain Ss are also infected with *Rickettsiella viridis* (Rv) since the P36 donor clone was co-infected by Ss and Rv (Simon et al. 2011). All aphid lines were established more than ten years ago and were tested for the presence of the expected FS before and during the experiments using previously described PCR methods (for a review see Desneux et al. 2018). Parthenogenetic aphids were maintained on *Vicia faba* at 20 °C and

16:8 h light/dark cycle. To obtain synchronized adults aged 13 to 14 days for the experiment, aphids were reared as described previously (Schmitz et al., 2012).

Hemolymph collection

Aphids (apterous or alate as indicated) were cleaned with a 70% ethanol bath, rinsed with distilled water, and immersed on a Petri dish on ice in a drop of 10 μ l Ringer's (KCl 182 mM; NaCl 46 mM; CaCl₂ 3 mM; Tris-HCl 10 mM) solution / aphid supplemented with a cocktail of protease inhibitors (Sigmafast; Sigma) for electrophoresis. The ventral cuticle was then carefully ruptured under a stereomicroscope and the diluted hemolymph was collected as described (Schmitz et al., 2012) and immediately centrifuged at 15,000g for 10 min. The supernatant and the whole pellet were carefully collected, mixed separately with Laemmli buffer containing β -mercaptoethanol, and boiled. Three biological replicates were done for each of the nine aphid lines used.

Hemolymph 2D gel separation and mass spectrometry

The hemolymph of 150 adults LL01 was collected by successive dissections in a drop of 150 μ l drop of Ringer's solution supplemented with a cocktail of protease inhibitors (Sigmafast; Sigma). After centrifugation (5 min, 15,000g, 4°C), the supernatant was treated for two-dimensional electrophoresis as previously described (Syntin et al. 1996). Briefly, the sample was mixed with 5% of solubilization solution (0.15M dithioerythritol, 10% SDS), boiled and cooled to room temperature before adding the same volume of urea solution (9.2 M urea, 2% Ampholytes [1% pH 3-10 (Pharmacia) and 1% pH 2-11 (Servalytes)] and 2% CHAPS). Samples were loaded onto a 5% acrylamide one-dimension gel tube (15cm) and, after overnight isoelectric focusing, the gel was incubated in 4x reducing Laemmli (Laemmli, 1970) and loaded on top of a 12% acrylamide gel. The gels were then silver stained (Morrissey 1981) or immunoblotted, as described further.

The major spots of the 2D silver stained gel were cut, rinsed and then reduced with dithiothreitol and alkylated with iodoacetamide. Samples were incubated overnight at 37°C with 10 ng/ μ L trypsin (sequencing grade; Promega) in 25 mM NH₄HCO₃. Protein identification was performed by Ion Trap LC-MS/MS (Exactive Q, Thermo-Fischer Scientific). Peptide and fragment masses were matched into a database (nrNCBI *A. pisum* or *B. aphidicola*) using the MASCOT software (<http://www.matrixscience.com>). The sequence validation criteria were (i) a peptide with an individual ion score greater than 50 (the Mascot significant identity threshold corresponding to $p < 0.05$ was 36) or (ii) at least two individual ion score peptides greater than 20 (corresponding to a 1% probability that a peptide spectrum corresponds to a random event).

The score was calculated as $-10\log(P)$. The analysis was performed with an ion mass tolerance for 0.02 ppm fragments and a parent ion tolerance of 10 ppm.

Total protein extraction from aphids with and without hemolymph

Ten adults were randomly selected from a synchronized pool of aphids and cleaned with ethanol as described. Five aphids were weighted, bled and their carcasses used for protein extraction (“bodies without hemolymph”). The other five aphids were weighted and used directly for the extraction of proteins (“whole bodies”). The extraction was done as follows: pooled aphids were placed in a 1.5 ml tube with 50 μ l of Ringer’s solution supplemented with protease inhibitors and 50 μ l of 4x Laemmli buffer. The aphids were crushed with a pestle and this extract was centrifuged at 15,000g for 10 min. The supernatant was carefully collected, then 5 μ l was mixed with 100 μ l of 2x reducing Laemmli buffer and the samples were boiled. For the 6 aphid lines used (LL01, YR2-Ri(n), YR2-Hd, YR2-Ss, YR2-Amp and YR2-Ri(a)), 3 biological replicates were performed.

Western blotting

To compare the amount of PO proteins in the hemolymph, the proteins were first separated on a 12.5 % SDS-PAGE and the gel was silver stained (Morrissey, 1981) to evaluate the total amount of proteins and equalize the samples. The adjusted protein amounts were loaded onto two 12.5 % SDS-PAGE: one was silver stained and photographed using a digital camera (EOS5D, MKII; Canon) and the other was transferred to a 0.2 μ m nitrocellulose membrane (Amersham). After the transfer, the membrane was blocked with TBS-Tween 2% low fat milk, and then incubated overnight at 4 °C with an in-house rabbit polyclonal antibody against aphid PO. The polyclonal antibody was directed against two peptides (QTGSRTPIEIPHDY and SMGYPFDRTPRDG) common to both aphid POs (see Fig S1). Peptides were coupled to BSA using Sulfo-NHS-LC-Biotin (Pierce) and immunization was done as previously described (Gatti et al. 1999). The serum obtained after the fourth injection was diluted in glycerol (50/50: v/v) and kept at -20°C. To probe the Western blot, it was used at a final dilution of 1/2500. After three washes in TBS, the membrane was incubated for 2 hours with goat anti-rabbit-HRP (1/10000; Sigma) at room temperature. The signal was revealed using a Luminata Crescendo Western HRP substrate (Merck Millipore) and digital pictures obtained with an imaging device (Chemi Genius²; SynGene). The total intensity of each lane of the silver stained gel and each reactive band on the Western blot was quantified using the Image J software, and the intensity of the Western blot reactive band was divided by the intensity of the corresponding silver stained lane to correct for the variation of the amount of loading between the lanes. The same protocol was used for the quantification of PO protein in “whole bodies” and “bodies without

hemolymph". The preimmune rabbit serum and the secondary antibody alone were not reactive on the hemolymph by Western blotting under the same conditions. To investigate whether PO was proteolyzed after collection, pooled hemolymph of 10 YR2-Amp, LL01 or T3-8V1-Amp adult aphids were collected in 100µl of water, centrifuged (10,000g, 1 min, 4 °C) and the supernatant was kept at room temperature. 10 µl were taken immediately and after 10, 30 and 60 min and processed for loading on gel and Western blot as described.

cDNA synthesis and semi-quantitative RT-PCR analysis

Total RNA was isolated from 5 pooled aphids using the RNeasy plus micro kit according to the manufacturer's instruction (Qiagen, Germany) and quantified on a nanodrop (Agilent). The cDNA was generated from 500ng RNA using the iScript cDNA synthesis kit (Hercules, California, USA), according to the manufacturer procedures. Specific mRNA primers (Table 1) were designed for RT-PCR to avoid genes amplification using Primer3 (Untergasser et al. 2012), for PO2 (ACYPI001367; XM_001949272.1; XP_001949307.1; previously ProPO1 (Gerardo et al., 2010)), PO2-X1 (ACYPI004484; XM_001951102.1; XP_001951137.1; previously ProPO2 (Gerardo et al., 2010)) and the reference gene Elongation Factor-1 (EF1; ACYPI006711-RA; XM_003243999.1_DG2). The PCR was carried out in 25µl containing 5µl of 5x green reaction buffer (Madison, Wi USA), 1µl of forward primer, 1µl of reverse primer, 2.5µl of 5mM MgCl₂, 0.5µl of 20mM dNTP, 0.125 µl of GoTaq DNA polymerase (Madison, Wi USA), 1.5µl of cDNA (100ng/µl) and nuclease-free water. The PCR cycle parameters were as follows: 95 °C for 4 min and 30 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, then 72°C for 10 min. The resulting PCR products were separated in a 1.5 % agarose gel with ethidium bromide and visualized under UV. The Image J software was used to obtain the intensity of the PCR bands. The intensity of the PO bands was divided by the intensity of the EF1 band of the same sample to estimate the level of expression of the PO gene. Controls were carried out by replacing the RT products with the same volume of H₂O in the final mix. All assays were done in triplicate on different biological samples.

Phenoloxidase (PO) activity

PO activity was tested through its catalytic conversion of colorless L-Dopa (3,4-dihydroxy-L-phenylalanine; Sigma) to brown-red dopachrome (Saul et al., 1987). Measurements were performed directly on the pooled hemolymph of five 14-day-old aphids. The hemolymph was collected directly into a drop of 70µL distilled water, which allowed the best activation of the PO. The diluted hemolymph was immediately transferred to a 0.5 mL microcentrifuge tube and centrifuged (10,000g, 1 min, 4 °C). The supernatant was collected and centrifuged again under the same conditions. Finally, 50µL were collected, immediately frozen in liquid nitrogen and

kept at -80°C until the PO activity test. Before use, samples were thawed on ice and vortexed. Then, 15µl of sample were mixed with 150µl distilled water (as a control) or 150µl of L-dopa solution (1mg/mL in distilled water; two technical replicates per sample) in a 96-well plate. The reaction was monitored at 30 °C in a spectrophotometer (Versamax, Molecular Devices) by reading at 490 nm every 30 seconds for 1 hour. Activity curves were analyzed using Softmax Pro v 4.0 software. The enzymatic activity (V_{max} in OD/min) was taken as the maximum linear slope during the conversion of the substrate. At least six biological replicates were done for all of the assays.

Fitness measurement under different challenges

These experiments being time consuming, lines from only YR2 clones were used. For the fitness test, the controls were performed with ten 13-day-old aphids of YR2-Amp, YR2-Ss and YR2-Hd that were transferred to a 9-cm Petri dish filled with moistened filter paper and containing a freshly cut leaf of fava bean with the stem in a wet cotton wrapped with aluminum foil. 24 hours later, we began recording the number of offspring (counted and removed) and the number of dead aphids each day until the death of all individuals. Leaves and filter papers were replaced every 3 days. The numbers of replications of all the tests in the study were at least 8-fold. Experimental conditions were 20 °C and 16:8 h light/dark cycle.

For stress tests, 13-day-old adult aphids from four lines (YR2-Amp, YR2-Ss, YR2-Ri(n) and YR2-Hd) were microinjected either with PBS (phosphate-buffered saline) as a control, polystyrene latex beads modified with fluorescent carboxylate (L3030; Sigma), live yeast (66A strain; from an OD₆₀₀ culture = 0.3) or *E. coli* (PFPV 25.1 strain; from an OD₆₀₀ culture = 0.3). We choose these latex beads because they had previously been shown to be phagocytized by aphids hemocytes just like *E. coli* (Schmitz et al. 2012). The injected volume was 69 nl (nanoinject II, Drummond Scientific Company). For each aphid line, aphid pools of 10 individuals for the measurement of fitness and 5 individuals for the PO were injected for each condition. The number of offspring and the number of live and dead aphids were recorded as describe above in at least 8 biological replicates. To investigate whether changes in the amount of PO protein, PO gene expression and PO activity occurred rapidly after injection, control and stressed aphids were placed on fava bean plants and then collected 24 hours post-injection and assays performed as described above.

Statistical analysis

All protein amounts, gene expression, and enzyme activity were analyzed by performing ANOVA after validation of the normal distribution of the dependent variable. Aphid survival was analyzed using the proportional hazards model (Cox 1972), and a set of survival measures

was used (Therneau 2015). The number of aphid offspring number was analyzed using general linear models after validation of the normal distribution of the dependent variable. The package ‘multcomp’ was used to perform multiple comparisons using Tukey’s contrasts. All statistics were performed in R version 3.4.3. All data and complementary information are provided in Supplementary File 1.

Results

Pea aphid POs are hemolymph proteins

In insects, phenoloxidases can be intracellular and/or free enzymes circulating in the hemolymph (Rizki and Rizki 1959; Cerenius and Söderhäll 2011; Dudzic et al. 2015). We first sought to identify which phenoloxidase(s) are present in the aphid hemolymph and determine whether they are associated to cells, circulating or both. The hemolymph of the LL01 aphid line (devoid of secondary symbiont) was used for the analysis of circulating proteins. The purified hemolymph (after removal of cells and debris by centrifugation) was processed and separated by 2D gel electrophoresis (Fig. 1) and 50 major spots were cut and analyzed using an ion trap LC-MS-MS. Table 2 shows the best protein hit for each spot with information on their possible functions (the missing spots have either no match in the database or are below the Mascot cutoff; the complete list of all proteins identified can be found in Table S1). The majority of these proteins are metabolic enzymes and cell-derived proteins, a non-unexpected result since the insect hemolymph is a close system that can also serve as a sink for removing cellular components. Interestingly, some proteins such as GroEL (spot 10) are derived from the obligate symbiont *B. aphidicola*. Since GroEL is also present in aphid saliva (Chaudhary et al. 2014), this suggests that the hemolymph could be used to transport this protein from bacteriocytes to salivary glands.

Phenoloxidase has been identified in spot 6, the best match being the variant X1 of PO2. The intensity of silver staining of these spots was low, suggesting that PO is not a quantitatively important hemolymph protein. The presence in these spots of the second PO protein (PO2) could not be ruled out since four of the nine identified peptides were common to both sequences (Fig. S1) and both enzymes have almost the same theoretical molecular weight of about 72 kDa. For antibody production, we thus designed peptides common to both sequences (Fig. S1). This antibody only recognized a single series of spots close to the expected size and position of the PO on the 2D gel (Fig. S2). Using this antibody, we checked whether immune cells circulating in the hemolymph could also contain a non-negligible amount of PO. On the Western blot, one

band at about 70 kDa was strongly reactive on lane containing pure hemolymph whereas none was visible on the cell pellet, suggesting a very small amount of PO in the hemocytes (Fig. 2).

Quantification of PO protein amounts in the aphid hemolymph

The amount of PO protein in the hemolymph was compared in LL01 and the different YR2 and T3-8V1 lines (Fig. 3) by combining silver stained gels and Western blotting quantification as described in Mat and Meth. The amount of PO protein was almost identical in the hemolymph of LL01, YR2-Amp and YR2-Ss and much higher than in YR2-Hd and YR2-Ri(n) (which was equivalent to YR2-Ri(a); not shown) (Fig 3A and B). A similar result was found in the T3-8V1 lines (Fig 3C and D), with an equivalent amount of PO in the hemolymph of T3-8V1-Amp and T3-8V1-Ss, much higher than in T3-8V1-Hd. When comparing directly LL01, YR2-Amp and T3-8V1-Amp, there was an effect of the genetic background, with about 5-fold more circulating PO protein in T3-8V1-Amp than in the other lines (Fig S3). Besides, the presence of the symbionts in the YR2 lines had the same effect on the amount of PO proteins in alate aphids as in non-alate ones (Fig S4).

To determine if all the PO proteins circulate in the hemolymph and, therefore, the difference observed would not be due to retention or storage of POs in certain lines, we compared the total amount of PO between intact and bled aphids. In the YR2 lines tested, the majority of PO proteins were removed from the aphids by bleeding (Fig S5), more than 70% in YR2-Ri(n) and YR2-Hd (where the total amount is less) and up to 80-90% in the other lines. Thus, the difference in the amount of PO in the hemolymph measured between lines seems to reflect very well the amount of total PO in the aphid.

In all the different lines, the amount of PO and the PO activity in the hemolymph were congruent (Fig S6). However, since some insect POs are synthesized under a pro-form requiring proteolytic activation to be enzymatically functional (González-Santoyo and Córdoba-Aguilar 2012), we checked whether this type of processing occurred in aphids under the conditions of our enzymatic assay. The hemolymph of LL01, YR2-Amp and T3-8V1-Amp was collected in water (as for activity testing), centrifuged to remove debris, and samples were tested by WB for one hour at room temperature. The protein visualized by silver staining or observed on Western blot after labeling with the PO antibody showed no modification or change in position, for all three lines (see result for YR2-Amp in Fig S7). This strongly suggests that aphids do not have the mechanism of controlling PO activity in the hemolymph by a cascade of serine proteases / inhibitors of serine proteases, as identified in other insects (Lu et al. 2014).

PO genes expression

Using the different lines, we tested the effect of the presence of facultative symbionts on the expression of the two PO genes (Fig 4; see also Fig. S8). The level of gene expression was similar in LL01 and YR2 lines without FS or with *S. symbiotica* but lower in the presence of *R. insecticola* or *H. defensa*. A similar result was found in T3-8V1 lines (Fig. 4) although the level of expression in T3-8V1-Amp was higher than in LL01 and YR2-Amp. In all lines, PO2 was at least twice as expressed as PO2-X1, strongly suggesting that both proteins are synthesized in pea aphids.

Aphid lines response to different biotic stresses

Injection of PBS had no effect on aphid survival compared to non-injected aphids (Fig S9) while among aphid lines, YR2-Hd and YR2-Ri survived less time compared to YR2-Amp and YR2-Ss (Fig. 5). By taking PBS injection as a control, the injection of almost all stressors reduced survival of all aphid lines (Fig. 5), the exceptions being YR2-Amp, not affected by the injection of yeast, and YR2-Ss, not affected by that of beads and yeast. Overall, i) aphids with *R. insecticola* or *H. defensa* survived less well (about 20% shorter lifespan) than YR2-Amp or YR2-Ss (Simon et al. 2011), ii) injection of *E. coli* was the most impacting stress (until 50% reduction of lifespan for certain lines), iii) beads and yeast surprisingly had a very similar effect (about 20% of life shortening) except for YR2-Amp for which yeast had no effect. It is recognized that increased production of offspring is one of the main aphid responses to stress, such as parasitism by wasps (Barribeau et al. 2010; Gerardo et al. 2010). Here, we compared the number of offspring per day per group of aphids to avoid the bias due to the death of part of the aphids during the experimental period. In agreement with published data (Simon et al. 2011), this number was lower in the presence of *R. insecticola* or *H. defensa* under unstressed conditions (Fig 5). Injection of PBS did not change the number of offspring compared to untreated aphids (Fig S9). Compared with PBS injection, all stresses slightly increased the number of offspring in all aphid lines, but this increase was significant only for the YR2-Ri(n) line injected with yeast. Overall these data suggest that having a higher level of circulating PO before artificial infection did not change the global effect of stressors on the aphid fitness.

24h after injection, the amount and activity of the PO protein increased in stressed YR2-Amp and YR2-Ss, but only significantly after injection of yeast. On the other hand, no significant change was observed for YR2-Ri(n) and YR2-Hd, nor for other stressors (Fig 6). We did not observe any significant difference for the level of PO activity between untreated aphids and those injected with PBS ($F_{1,14} = 0.0328$, $P = 0.859$; $n = 8$) indicating no effect of the injection itself. Surprisingly, although correlated with each other, the expression of the genes was not consistent with the amount and activity of the proteins (Fig 6 and 7). Compared to PBS, the

injection of beads and yeast, but not that of *E. coli*, significantly lowered the level of expression of PO genes in YR2-Amp, whereas the same effect was observed in YR2-Ss and YR2-Ri, also with *E. coli*. For YR2-Ri, only the expression of PO2 was affected. For YR2-Hd, the only effect was a strong increase in the level of expression of both genes after injection of *E. coli*. The injection of *E. coli* had therefore no effect in YR2-Amp, reduced the level of expression of PO genes in YR2-Ss and -Ri(n) but increased it in YR2-Hd. Overall, the level of expression was thus strongly dependent on the FS species hosted and the injected stress factor.

Discussion

Although the pea aphid is used as a model organism to study symbiosis, our knowledge of the interaction between its immune system and hosted symbionts remains scarce. Previously, we showed that the number of adherent immune cells and the PO activity in the total hemolymph were lower in the presence of *R. insecticola* and *H. defensa*, but not *S. symbiotica*, compared with those of the same clones without facultative symbionts (Schmitz et al. 2012). However, the mechanism(s) involved has not been explored further. Here, we focused on pea aphid phenoloxidases, identifying the PO protein(s) in the hemolymph by proteomic and Western blotting, but we could not unambiguously determine if only one or both of the predicted PO proteins (PO2 and PO2-X1) were present. Indeed, we could not separate the two proteins that have almost the same MW and are not processed in the hemolymph by proteases, as in other insects, which could have helped to differentiate them. This absence of cleavage nevertheless suggests that pea aphid POs are self-activated, possibly by a mechanism similar to that used by *D. melanogaster* PPO3 (Liu et al. 2012). The fact that both PO genes are expressed in all aphid lines tested (PO2 being twice as high as PO2-X1) suggests that both proteins are synthesized. The expression of the two genes has previously been reported (Gerardo et al., 2010; Xu et al. 2019) and, interestingly, blasting PO2 or PO2-X1 on aphids sequences in NCBI resulted in matches with two PO sequences for each of the different aphid species in the database. The clustering of each of the sequences with one of the pea aphid PO suggests a conserved ancestral duplication.

Our data indicate that almost all of the PO in adult aphids was circulating, with PO being undetectable by Western blot in hemocyte pellets. Our previous studies showed that only about 3% of adherent hemocytes exhibited phenoloxidase activity (Schmitz et al. 2012), this small number of cells being possibly responsible for the lack of immunodetection. Unlike the pea aphid, the body cavity of soldier nymphs of the social aphid *Nipponaphis monzeni* contains large hemocytes filled with high amounts of lipid droplets and rich in phenoloxidase (PO).

These cells are expelled during defensive behaviors to protect the gall of the aphid: the expelled hemocytes break down and release lipids and PO that converts tyrosine into reactive quinones that crosslink proteins to physically reinforce the clot to close the hole in the gall (Kutsukake et al. 2019). In this study, the authors also reported that pea aphid hemocytes are enriched in PO mRNA compare to the whole body. We did not observe a difference in the amount of POs mRNA between whole and bled aphids, suggesting that the majority of POs mRNA are not associated with hemocytes (not shown). Although our data suggest that hemocytes are not the primary source of PO protein in pea aphids, additional studies will be required to conclude definitively: Some non-adherent hemocytes, such as spherulocytes involved in the clotting process or wax cells expelled by the cornicles (Schmitz et al, 2012), may contain POs and lyse rapidly like *Drosophila* crystal cells during hemolymph collection (Rizki and Rizki 1959; Banerjee et al. 2019).

The comparison between clones of aphids without secondary symbionts (LL01, YR2-Amp, T3-8V1-Amp) indicates that the genetic background influences the PO level: T3-8V1-Amp shows much higher basal expression, quantity, and activity of PO than the other two clones for which they were almost equivalent. In both genetic backgrounds tested (YR2 and T3-8V1) all of these PO traits were decreased in the presence of *H. defensa* and *R. insecticola* in YR2, but not of *S. symbiotica* (and *Rickettsiella viridis*). This was observed with the *R. insecticola* natural strain (Ri^{YR2}) whereas the effect of the natural strain (Ri^{T3-8V1}) in T3-8V1 and of (Ri^{T3-8V1}) artificially introduced into YR2 was much lower, suggesting an effect of the genotype of the symbiont on the magnitude of negative effects on PO. In the different lines, the expression of the PO genes, the amount of PO protein and the PO activity correlated well. Thus, the presence of certain facultative symbionts would selectively affect the level of innate immune components of *A. pisum*. This effect occurs primarily at the level of gene expression, with a reduction of at least 50%, but also at the level of protein production since this amount was reduced by more than 80%.

In aphids, the essential genes for five non-essential amino acid pathways absent in *Buchnera* are upregulated in primary bacteriocytes containing *Buchnera*, indicating a regulatory integration of genes between the two partners (Xie et al. 2010; Ballinger and Perlman 2019). Facultative symbionts also provide new phenotypes to the host by various means. An example is the protection against parasitism conferred to *Drosophila melanogaster* by the presence of certain strains of *Spiroplasma*. The bacterium can compete for specific lipids with the parasitoid larva, killing it by depriving it of these essential metabolic compounds (Xie et al. 2010). In the case of aphids, protection against parasitoid wasps provided by *H. defensa* varies among strains

differentially infected by APSE bacteriophages (Oliver et al. 2009). This protection is associated with the production and secretion of toxins targeting eukaryotes (Repeats-in-Toxin (RTX) Toxins) homologs to the Shiga-like toxin (Stx) and cytolethal distending toxin (CdtB) present in the genomes of some APSE strains (Degnan et al. 2009). RTX toxins, originally classified as leukotoxins and hemolysins, have been shown to penetrate and permeabilize host cell membranes. Some of the RTX toxins act on the signaling of host cells to alter cellular physiology, others induce cytoskeletal rearrangements or trigger an apoptotic program on the targeted cells. *Regiella insecticola* is a sister species of *H. defensa*, the two bacterial genomes being divergent but still having ~55% of genes in common (Rollat-Farnier et al. 2015). The functions encoded by these shared genes include host cell toxicity and invasion capabilities (type 3 secretion systems and RTX toxins). *Serratia* species, including cultivable strains of *S. symbiotica*, secrete extracellular proteins (e.g., proteases, lipases, DNases and chitinases) that can modulate their environment to their advantage. However, the genome of *S. symbiotica* of the pea aphid has shown widespread pseudogenization and inactivation of pathogenesis genes (Burke and Moran 2011). In addition, the heat stress tolerance it provides to the host does not come from the secretion of extracellular products but from bacterial lysis that releases protective metabolites (Kong et al. 2008). We therefore hypothesize that some of the common toxins secreted by *H. defensa* and *R. insecticola*, and absent from *S. symbiotica*, may affect PO production by targeting the producer cells. Currently, we do not know where the immune cells and the POs come from in aphids. In *Drosophila*, PO originates from certain hemocytes and hematopoiesis is a complex mechanism modulated by various conditions (Letourneau et al. 2016; Kim-Jo et al. 2019; Banerjee et al. 2019): the embryonic hemocyte lineage forms pro-hemocytes that multiply and differentiate to produce the different types of hemocytes either directly in the hemolymph or in specific organs during the larval development (lymph gland and sessile compartments), some of these produced cells remaining in the adult. The timing and the type of cells produced will also depend on the pathogenic conditions encountered by the larvae (Letourneau et al. 2016; Kim-Jo et al. 2019; Banerjee et al. 2019).

We also tried to stimulate a PO response in the different YR2 lines by biotic and abiotic challenges and we measured their impacts on some life history traits (e.g., offspring number and survival). With the exception of yeast in YR2-Amp, almost all treatments induced a reduction in lifespan in YR2 lines, with the greatest amplitude produced by *E. coli* as previously reported (Altincicek et al. 2011). Having a higher basal PO level did not dramatically change the survival of aphids after different stresses. In contrast, after stress, only a slight increase in individual reproduction was observed during life-time compared to the control (whereas the

overall number of offspring was reduced due to shorter aphid life). Fertility compensation was considered an effective response to immune challenges for organisms such as aphids with limited immunity (Altincicek et al., 2008; Barribeau et al., 2010). Our results showed that the presence of symbionts and the type of stress can temper this response.

The activity and amount of PO increased only in YR2-Amp and YR2-Ss (two lines with already high basal PO) in response to yeast injection. Surprisingly, this was not due to an increase in gene expression since the level of transcripts for the two POs decreased 24 hours after infection. None of the other treatments has a significant impact on the amount and activity of PO but all reduced the amounts of mRNAs except *E. coli* in YR2-Hd. In the short term after the stress, there is therefore no apparent correlation between the total expression of PO genes and the amount of PO protein in the hemolymph. The increase in PO activity and the amount of protein observed in YR2-Amp and YR2-Ss after yeast injection is therefore difficult to explain since we did not detect any “reserve” of PO in these lines. The only explanation is that the PO mRNAs present may be rapidly transcribed into PO proteins before their amount was reduced in these aphid lines under yeast stress or that the expression of PO increases transiently very rapidly (a few hours) after the stress. It would be very interesting to determine whether the effect of the presence of *Hd* or *Ri* is specific of the transcription of the PO genes or is due to an effect on the overall transcription in the cells producing the PO.

In conclusion, we demonstrate that phenoloxidase levels vary with the genotype of the aphid and are strongly affected by the presence of the facultative symbionts *H. defensa* and *R. insecticola*. These bacteria could control the expression and synthesis of both aphid POs through their retained pathogenic capacities, particularly the production of toxins. There is growing evidence of the microbial generation of bioactive compounds that impact the transcriptional machinery in host cells, including DNA methylation, histone modification and non-coding RNAs (Krautkramer et al. 2016; Qin and Wade 2017). Future works will therefore be needed to determine where (presence of a hematopoietic organ?) and when the POs and immune cells are produced during pea aphid development in order to identify the precise mechanism by which these FS might act.

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Tables

Table 1. Primers used in the study

Target	Primer name	Primer sequence 5' to 3'	Amplicon size (bp)
Phenoloxidase 2 (PO2)	PO2-paF	GGCAGTGGTTCAAACAGGTT	173
	PO2-paR	TTACGATATCCACCGGTCCG	
Phenoloxidase 2-X1 (PO2-X1)	PO2-X1-paF	CTTGTTTCGATCGTCCTACCG	194
	PO2-X1-paR	CCCAGTGGAATCGACAAGTC	
Elongation factor 1 (EF1)	EF1-paF	GGCCGACGGTAAATGTTTGA	232
	EF1-paR	CAAAGCTTCGTGGTGCATCT	

Table 2. Main protein identified in each 2D spot. Missing spot numbers indicated either no protein identification or protein under the Mascot score.

Spot n°	MW	Protein name	General function from UniProt and different literature sources.
1	96.9	Inter-alpha-trypsin inhibitor heavy chain H3 isoform	May act as a carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix protein, including those on cell surfaces in tissues to regulate the localization, synthesis and degradation of hyaluronan which are essential to cells undergoing biological processes.
2	89.2	Transitional endoplasmic reticulum ATPase TER94	Necessary for the fragmentation of Golgi stacks during mitosis.
5	89.5	Alpha, alpha-trehalose-phosphate synthase	Trehalose synthesis involves the transfer of glucose from UDP-glucose to glucose 6-phosphate to form trehalose-6-phosphate and UDP <i>via</i> the cytosolic trehalose-6-phosphate synthase.
6	80	Phenoloxidase (XP_001951137.1)	Phenoloxidases (POs) which can hydroxylate tyrosine (enzyme EC 1.14.18.1) and also oxidize o-diphenols to quinones (enzyme EC 1.10.3.1) are involved in melanization and nodulation. POs are copper-containing oxidoreductase enzymes, oxidizing phenolic compounds.
7	71.4	Heat shock 70 kDa protein cognate 4	The chaperone 70-kDa Hsp family is composed of heat-inducible proteins (Hsp70), which are expressed under cellular stress conditions, and heat shock cognate proteins (Hsc70), which are constitutively expressed without any stress stimulation. Hsc70 contains signal peptides which allow its nucleolar or its cytoplasmic localization, but may be secreted in certain circumstances.
8	67.3	Transketolase-like protein 2	Transketolases are cytoplasmic enzymes that catalyze the conversion of sedoheptulose 7-phosphate and D-glyceraldehyde 3-phosphate to D-ribose 5-phosphate and D-xylulose 5-phosphate. This reaction links the pentose phosphate pathway with the glycolytic pathway.
9	67.8	V-type proton ATPase catalytic subunit A	Catalytic subunit of the peripheral V1 complex of vacuolar ATPase. V-ATPase vacuolar ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells.
10	57.9	GroEL protein	Chaperone. Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
11	58	Lactase-phlorizin hydrolase-like	LPH contains two active sites. One hydrolyses the P-galactoside lactose. It hydrolyses also with much less efficiency other natural P-glycosides like cellobiose, cellotriose, cellotetrose and cellulose. The other active site hydrolyses P-glycosides with large hydrophobic alkyl chains like those in galactosyl- and glycosyl-p-ceramides.
12	49.8	Tubulin alpha-1 chain	Tubulin is the major constituent of microtubules.
17	46.7	Eukaryotic initiation factor 4A	ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to ribosome.
18	47.2	Enolase isoform X2	Enolase catalyzes the reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate as part of the glycolytic and gluconeogenesis pathways.
19	41.8	Actin-42A-like	Actins are highly conserved proteins involved in various cellular functions such as cytoskeleton structure, cell mobility, chromosome movement and muscle contraction.
22	43.1	Serine protease inhibitor 4	Serpins inhibit members from one of several peptidase clans, including chymotrypsin-like enzymes, subtilase-like proteases, papain-related cysteine proteases and caspases.
23	39.8	Fructose-bisphosphate aldolase	Fructose-bisphosphate aldolase, often just aldolase, is an enzyme catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P).
28	34.1	Regucalcin-like	Regucalcin (RGN) is a calcium (Ca ²⁺)-binding protein which plays an important role in the regulation of Ca ²⁺ homeostasis. May be secreted.
29	35.6	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Catalyzes the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). It has uracil DNA glycosylase activity in the nucleus. The human protein contains a peptide that has antimicrobial activity.
30			
31			
32	33.2	Spermidine synthase-like	Catalyzes the production of spermidine (a polyamine compound having various metabolic functions) from putrescine and decarboxylated S-adenosylmethionine (dcSAM).
33	28.9	Phosphoglycerate mutase	Catalyzes one of the terminal steps of the glycolytic pathway, the interconversion of 2-phosphoglycerate and 3-phosphoglycerate.
35	28.2	14-3-3 protein zeta	14-3-3 proteins are ubiquitous molecular chaperones with important roles through interactions with a multitude of binding partners including kinases, phosphatases, and transmembrane receptors.
36	32.2	Inorganic pyrophosphatase	Catalyzes the hydrolysis of inorganic pyrophosphate (PPi) forming two phosphate ions. Cytosolic enzymes that may be secreted in exosomes.
37	27.1	Triosephosphate isomerase-like	Triosephosphate isomerase (TIM) catalyzes the reaction to convert dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, and vice versa. TIM is the fifth enzyme in the eukaryotic glycolysis pathway.
38	19.7	Nucleoplasm	Acts as a chaperone for histones and thus regulates the assembly of nucleosome cores.
39	24.9	Peroxioredoxin-6-like	Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively.
46	20.9	ACYPI005249	Protein of unknown function (DUF4446); peptide signal predicted
47	19.3	ACYPI000294	Protein of unknown function; LPP20 lipoprotein like-domain; peptide signal predicted
48	19.3	ACYPI000294	Protein of unknown function; LPP20 lipoprotein like-domain; peptide signal predicted
49	17.9	ACYPI007294	Protein of unknown function; peptide signal predicted

Figures

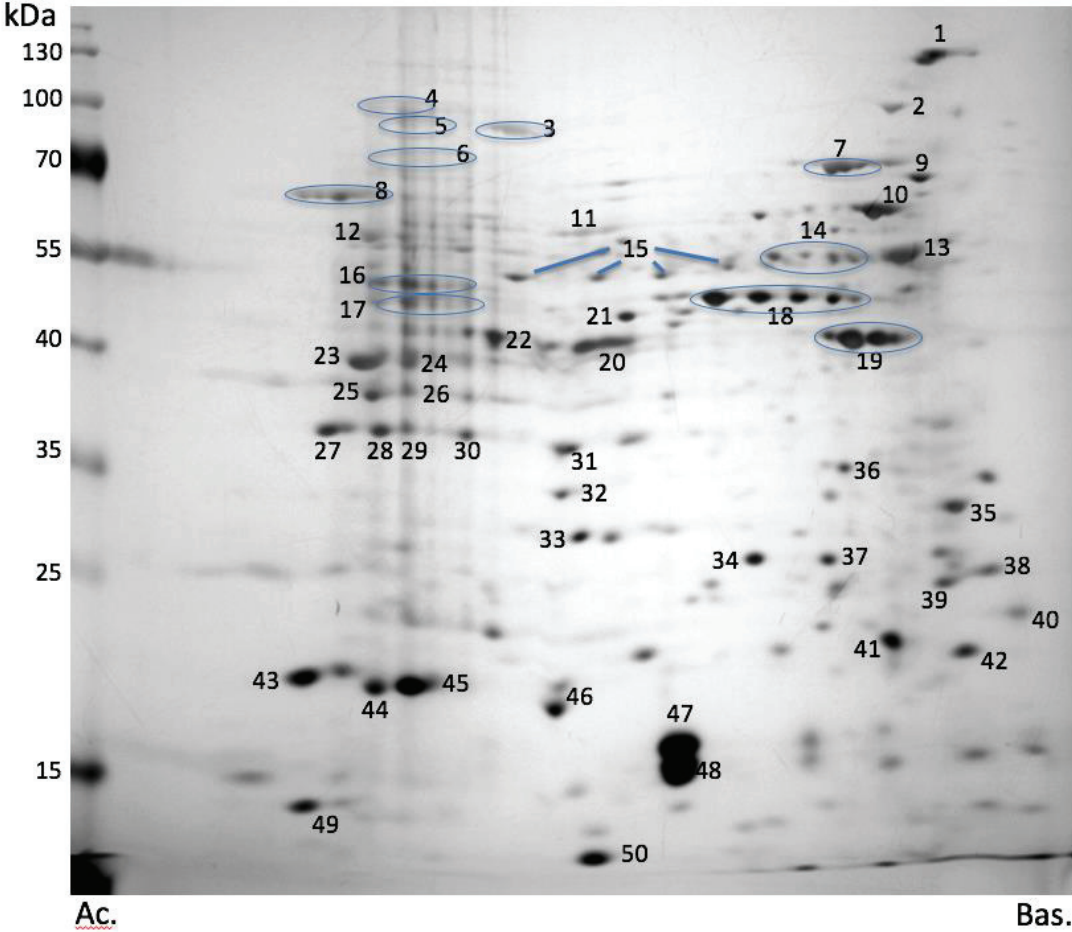


Figure 1. Two-dimensional gel electrophoresis of purified LL01 hemolymph. Picture of a 12.5% SDS-PAGE silver-stained gel of the purified hemolymph from LL01 aphids. The numbered spots are those cut for mass spectrometry analysis. Phenoloxidase was found in spot 6. MW in kDa. Ac indicates the acidic side of the gel; Bas, the basic side.

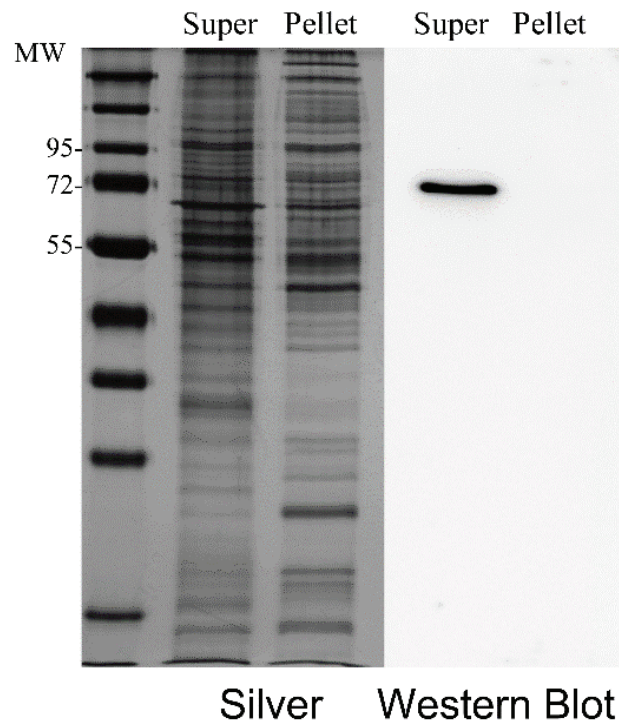


Figure 2. POs are mainly circulating in hemolymph. The hemolymph collected from 10 LL01 aphids was centrifuged at 15,000g and the supernatant (Super) and pellet (Pellet) proteins were separated on a 12.5% SDS-PAGE and silver stained (Silver). An equivalent gel was transferred to nitrocellulose and probed with the anti-PO antibody (Western blot). A single reactive band larger than 70 kDa was observed in the supernatant. The silver stained gel showed no signal in the pellet, this not being due to a lack of protein

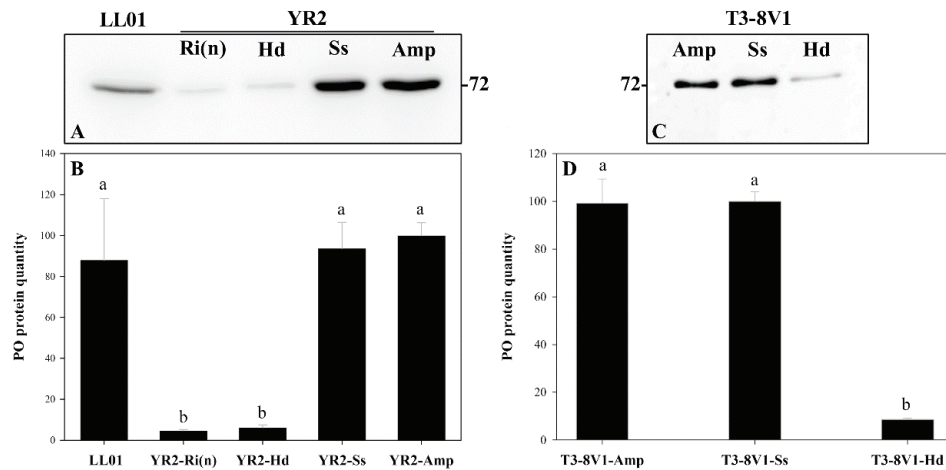


Figure 3. Comparisons of PO protein amounts in the hemolymph of the different aphid lines. PO protein revealed by Western blot using the same amount of hemolymph proteins for LL01 and the different YR2 lines (A), and the different T3-8V1 lines (C). The amount of PO protein was calculated as described in mat and meth for the LL01 and YR2 aphid lines (B) and the different T3-8V1 lines (D). For comparison, in B and D, values were set at 100% for the highest measured amount of PO in each aphid background (YR2-Amp for YR2/LL01 lines and T3-8V1-Ss for T3-8V1 lines, respectively). Results expressed as mean \pm SEM. Different letters (a, b) indicate significant differences ($p < 0.05$; multiple comparison test from “multcomp” package), $n = 3$.

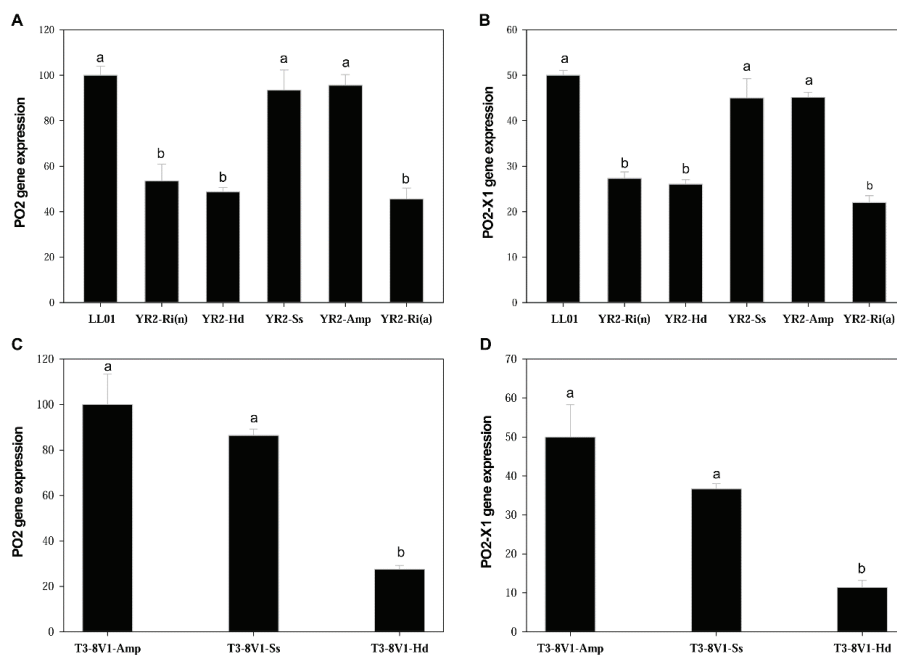


Figure 4. Expression of PO2 and PO2-X1 genes in the different aphid lines. The expression of PO2 (A, C) and PO2-X1 (B, D) genes was estimated in LL01 and the different YR2 (A, B) and T3-8V1 aphid lines (C, D). Gene expression values based on the ratio of PO2 or PO2-X1 intensity divided by the intensity of the reference gene EF1 on the same sample (see also supplementary figure 8). For comparison, the values were set to 100% for the highest measured expression of PO2 or PO2-X1 genes in each experiment (LL01 for LL01 and YR2 lines and T3-8V1-Amp for T3-8V1 lines). Results expressed as mean \pm SEM. Different letters (a, b) show significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 3$.

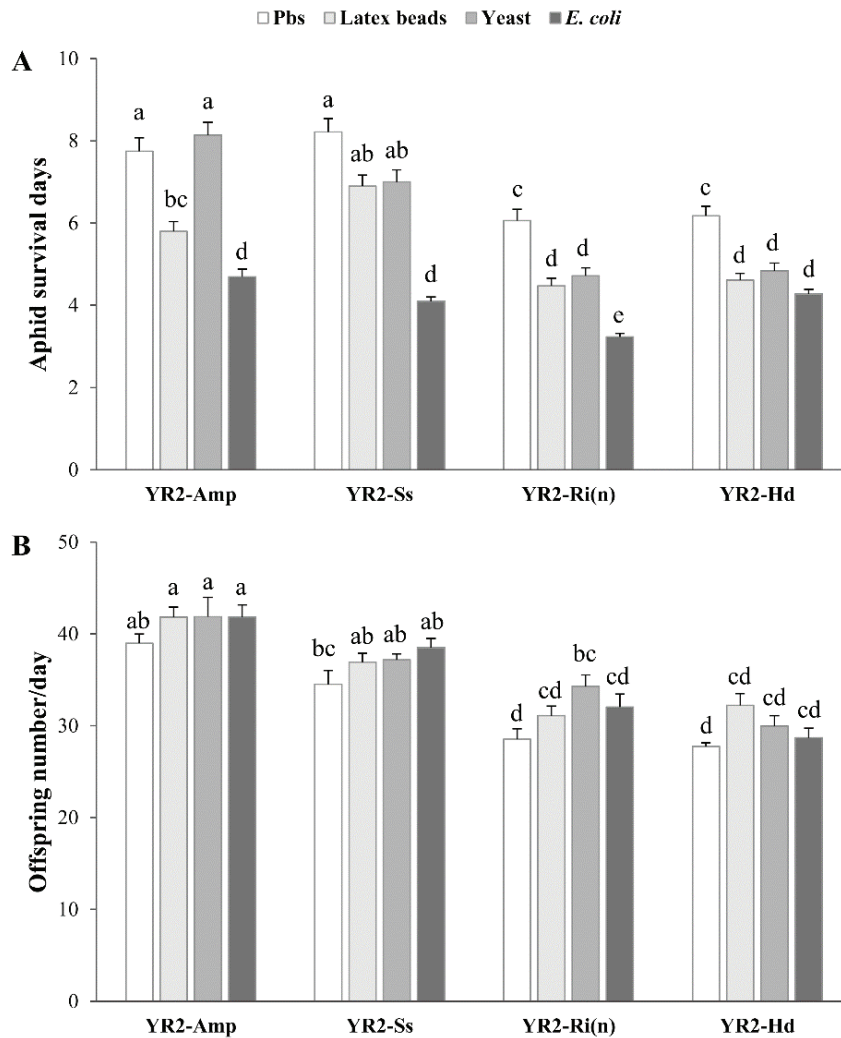


Figure 5. Changes in life-history traits of YR2 aphid lines after the induced stresses. The average number of aphid survival days (A) and the number of offspring produced per day per aphid pool (B) were estimated after microinjection of latex beads, yeast and *E. coli*. Microinjection of PBS was used as a control. Results expressed as mean +/- SEM. Different letters (a, b, c, d, e) show significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n > 8$.

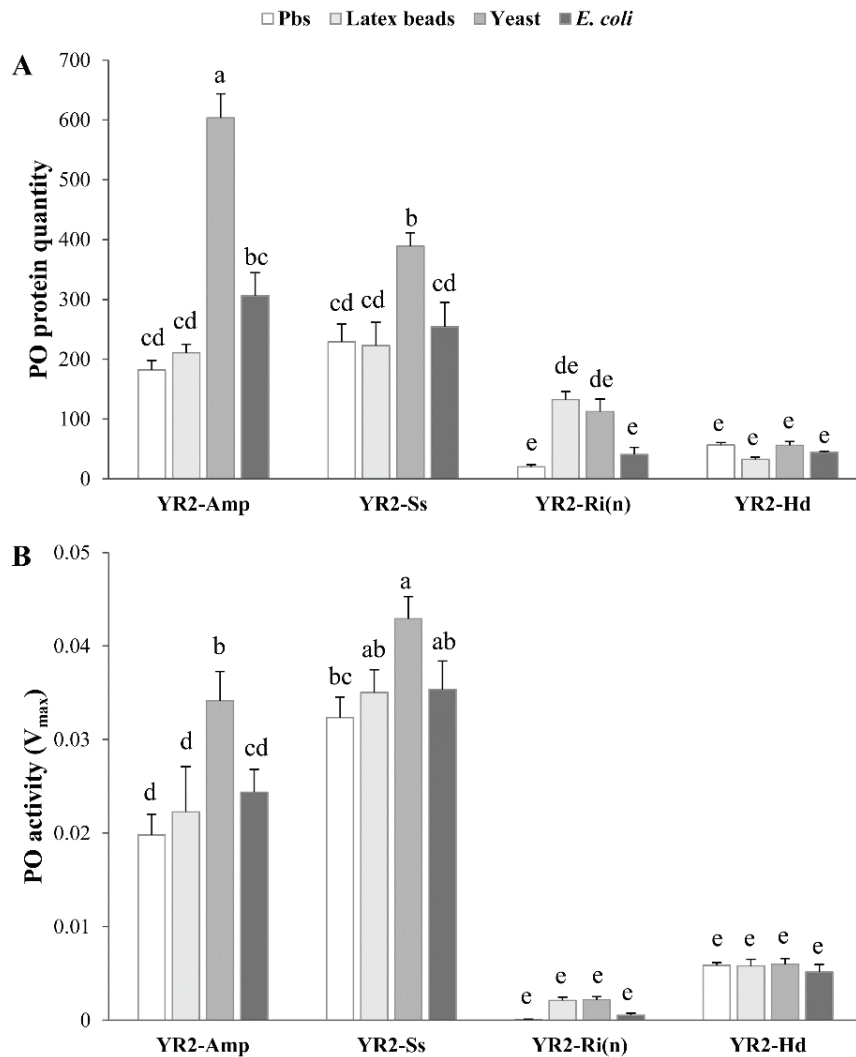


Figure 6. Changes in the amount and activity of PO in the hemolymph of YR2 lines after induced stresses. The amount of PO protein in aphid hemolymph was estimated by Western blot (A) and the PO activity measured by transformation of L-DOPA (B), 24 hours after microinjection of latex beads, yeast or *E. coli*. Microinjection of PBS was used as a control. Results expressed as mean \pm SEM; Different letters (a, b, c, d, e) show significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 3$ for PO protein quantity and $n > 6$ for PO activity.

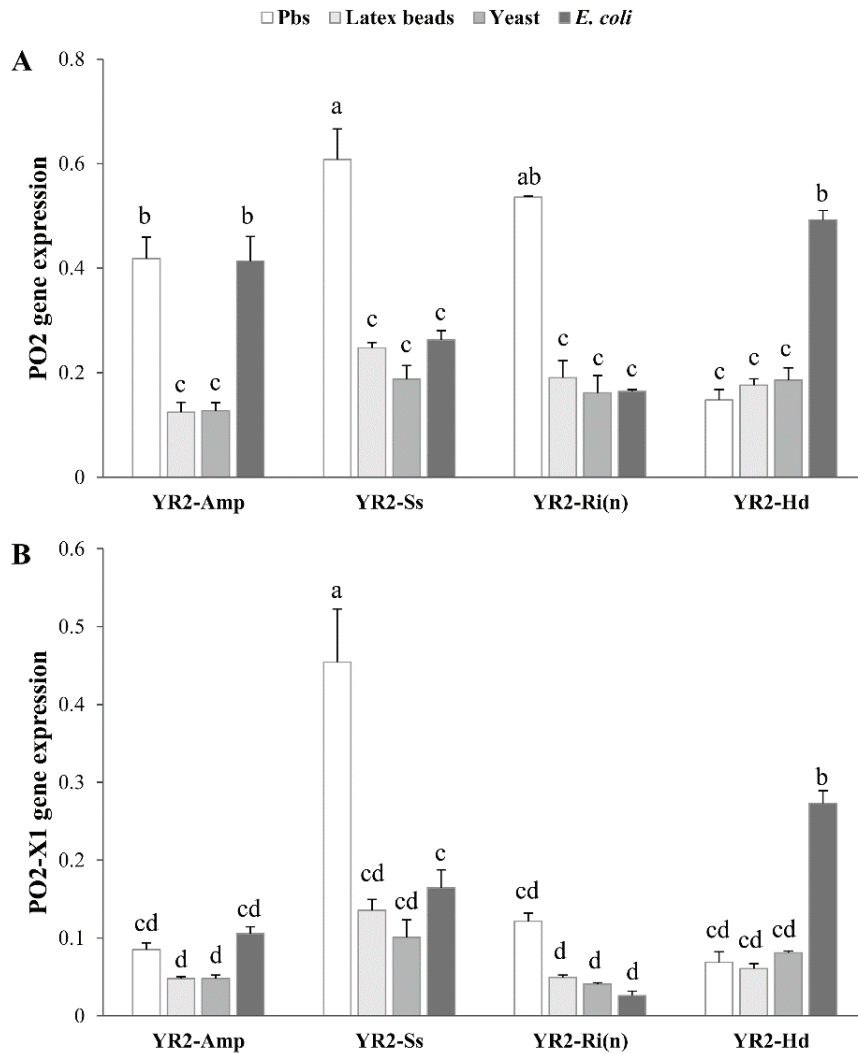


Figure 7. Changes in the expression of PO2 and PO2-X1 genes after induced stresses. The expression of the PO2 (A) and PO2-X1 (B) genes was estimated 24 hours after microinjection of latex beads, yeast or *E. coli*. Microinjection of PBS was used as a control. Results expressed as mean \pm SEM. Different letters (a, b, c, d) indicate significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 3$.

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Supporting Information

Table S1. Total protein identified ordered by their Mascot score.

Rank	Identification	Score	Mass	Matches	Sequences	emPAI	Name
1	sp B8D6T6.1 CH60_BUCAT	2098	57874	77 (60)	28 (21)	4.39	chaperonin GroEL [Buchnera aphidicola]
2	ref XP_008179407.1	1364	67772	42 (35)	20 (15)	2.23	PREDICTED: V-type proton ATPase catalytic subunit A [Acyrtosiphon pisum]
3	ref XP_001948685.1	1237	49865	46 (38)	16 (15)	2.67	PREDICTED: tubulin alpha-1 chain [Acyrtosiphon pisum]
4	ref XP_001944790.2	918	67346	45 (33)	21 (16)	1.63	PREDICTED: transketolase-like protein 2 [Acyrtosiphon pisum]
5	dbj BAH72928.1	838	35599	60 (30)	15 (7)	1.02	ACYPI009769 [Acyrtosiphon pisum]; glyceraldehyde-3-phosphate dehydrogenase
6	ref XP_001951207.1	762	71398	55 (36)	24 (16)	1.90	PREDICTED: heat shock 70 kDa protein cognate 4 [Acyrtosiphon pisum]
7	dbj BAH71065.1	614	27109	24 (20)	10 (6)	1.50	ACYPI006727 [Acyrtosiphon pisum]; Triosephosphate isomerase
8	gb ABD72705.1	500	41795	22 (14)	8 (4)	0.41	putative actin [Acyrtosiphon pisum]
9	ref XP_001943309.2	478	58043	27 (19)	15 (11)	1.11	PREDICTED: lactase-phlorizin hydrolase-like [Acyrtosiphon pisum]
10	dbj BAH72613.1	415	28208	17 (14)	7 (6)	1.42	ACYPI003154 [Acyrtosiphon pisum]; 14-3-3 protein epsilon [Acyrtosiphon pisum]
11	dbj BAH70597.1	343	23580	14 (8)	11 (7)	1.86	ACYPI002155 [Acyrtosiphon pisum]; PREDICTED: MICOS complex subunit mic60-like [Acyrtosiphon pisum]
12	gb ADI24740.1	334	19279	21 (15)	11 (7)	2.57	hypothetical protein [Acyrtosiphon pisum]
13	ref XP_001946187.1	325	57622	12 (6)	12 (6)	0.46	PREDICTED: T-complex protein 1 subunit beta [Acyrtosiphon pisum]
14	dbj BAH71207.1	322	22727	11 (8)	10 (7)	1.96	ACYPI009552 [Acyrtosiphon pisum]; Nascent polypeptide-associated complex subunit alpha
15	ref XP_008182079.1	322	60931	20 (14)	11 (9)	0.81	PREDICTED: phosphoglucomutase [Acyrtosiphon pisum]
16	gb ADI24738.1	315	50181	19 (11)	12 (8)	0.91	tubulin beta-1 chain [Acyrtosiphon pisum]
17	ref XP_016655758.1	295	51977	12 (6)	9 (4)	0.32	PREDICTED: alpha-enolase isoform X1 [Acyrtosiphon pisum]
18	ref XP_001945671.1	291	64895	16 (6)	14 (6)	0.40	PREDICTED: bifunctional purine biosynthesis protein PURH [Acyrtosiphon pisum]
19	ref XP_001943172.1	244	83365	39 (11)	20 (6)	0.30	PREDICTED: heat shock protein 83 [Acyrtosiphon pisum]
20	dbj BAH71727.1	229	32204	9 (6)	6 (4)	0.56	ACYPI006609 [Acyrtosiphon pisum]; Putative inorganic pyrophosphatase
21	ref XP_001951137.1	197	80051	19 (10)	12 (8)	0.50	PREDICTED: phenoloxidase 2 [Acyrtosiphon pisum]
22	dbj BAH71128.1	192	24903	20 (11)	10 (6)	1.71	ACYPI009972 [Acyrtosiphon pisum]; Putative Peroxiredoxin
23	ref XP_008184491.1	170	54544	10 (6)	9 (6)	0.49	PREDICTED: cytosolic non-specific dipeptidase [Acyrtosiphon pisum]
24	ref XP_003240405.1	155	39846	11 (4)	11 (4)	0.43	PREDICTED: fructose-bisphosphate aldolase [Acyrtosiphon pisum]
25	dbj BAH70816.1	152	29127	5 (3)	4 (2)	0.28	ACYPI006186 [Acyrtosiphon pisum]; Elongation factor 1 -delta
26	dbj BAH70707.1	147	33212	9 (7)	5 (3)	0.38	ACYPI006984 [Acyrtosiphon pisum]; spermidine synthase-like [Acyrtosiphon pisum]
27	ref XP_001943068.1	144	59917	9 (3)	9 (3)	0.20	PREDICTED: T-complex protein 1 subunit alpha [Acyrtosiphon pisum]
28	ref XP_003246082.1	143	55280	10 (4)	8 (4)	0.30	PREDICTED: V-type proton ATPase subunit B [Acyrtosiphon pisum]
29	dbj BAH71220.1	142	34748	6 (2)	4 (2)	0.23	ACYPI008104 [Acyrtosiphon pisum]; uncharacterized protein LOC100167297
30	dbj BAH70635.1	140	19724	10 (9)	4 (3)	1.04	ACYPI001342 [Acyrtosiphon pisum]; nucleoplamin isoform X1 [Acyrtosiphon pisum]
31	dbj BAH72093.1	140	44224	7 (5)	4 (2)	0.18	ACYPI000919 [Acyrtosiphon pisum]; cystathionase-like [Acyrtosiphon pisum]
32	dbj BAH70785.1	139	36750	16 (8)	9 (7)	0.98	ACYPI005685 [Acyrtosiphon pisum]; aldo-keto reductase-like [Acyrtosiphon pisum]
33	ref XP_001942978.2	126	67716	15 (6)	13 (6)	0.38	PREDICTED: moesin/ezrin/radixin homolog 1 isoform X1 [Acyrtosiphon pisum]
34	ref XP_008178940.1	124	45245	8 (2)	7 (1)	0.08	PREDICTED: serine protease inhibitor 4, serpin-4-like isoform X1 [Acyrtosiphon pisum]
35	ref XP_008179802.2	124	68326	14 (6)	12 (6)	0.37	PREDICTED: glycogen [starch] synthase [Acyrtosiphon pisum]
36	dbj BAH71334.1	120	19104	3 (2)	3 (2)	0.44	ACYPI006815 [Acyrtosiphon pisum]; uncharacterized protein LOC100165901
37	dbj BAH70530.1	116	34451	4 (3)	4 (3)	0.37	ACYPI000079 [Acyrtosiphon pisum]; ribosomal protein LP0 [Acyrtosiphon pisum]
38	ref XP_001947071.2	116	70406	13 (7)	10 (5)	0.29	PREDICTED: polyadenylate-binding protein 4-like [Acyrtosiphon pisum]
39	dbj BAH71077.1	114	36069	5 (4)	4 (3)	0.35	ACYPI004192 [Acyrtosiphon pisum]; guanine nucleotide-binding protein subunit beta-like protein [Acyrtosiphon pisum]
40	dbj BAH70525.1	110	25564	6 (3)	6 (3)	0.52	ACYPI005467 [Acyrtosiphon pisum]; uncharacterized protein LOC100164449

41	ref XP_001951017.1	103	33742	2 (2)	2 (2)	0.24	PREDICTED: pyridoxal kinase [Acyrtosiphon pisum]
42	ref XP_001950741.1	103	34065	2 (1)	2 (1)	0.11	PREDICTED: uncharacterized protein LOC100164964 isoform X1 [Acyrtosiphon pisum]
43	dbj BAH70568.1	103	31298	3 (2)	3 (2)	0.26	ACYPI002360 [Acyrtosiphon pisum]; uncharacterized protein LOC100161110 isoform X1
44	dbj BAH71185.1	100	31464	2 (1)	2 (1)	0.12	ACYPI004672 [Acyrtosiphon pisum]; omega-amidase NIT2-like [Acyrtosiphon pisum]
45	ref XP_0016662161.1	95	78296	4 (3)	3 (3)	0.15	PREDICTED: L-asparaginase [Acyrtosiphon pisum]
46	ref XP_001946818.1	91	59824	10 (3)	8 (3)	0.20	PREDICTED: T-complex protein 1 subunit theta [Acyrtosiphon pisum]
47	ref XP_001950362.2	91	75322	4 (2)	3 (2)	0.10	PREDICTED: putative ATP-dependent RNA helicase P110 [Acyrtosiphon pisum]
48	ref XP_003242240.1	89	53324	7 (2)	5 (1)	0.07	PREDICTED: hexokinase type 2 isoform X3 [Acyrtosiphon pisum]
49	emb CAQ65015.1	88	30003	10 (6)	4 (3)	0.43	elongation factor 1-alpha, partial [Acyrtosiphon pisum]
50	ref XP_008183814.1	87	45784	1 (1)	1 (1)	0.08	PREDICTED: aspartate aminotransferase, cytoplasmic [Acyrtosiphon pisum]
51	ref NP_001153852.1	86	120044	9 (1)	9 (1)	0.03	ATP citrate lyase [Acyrtosiphon pisum]
52	ref XP_001949588.1	84	89230	30 (8)	14 (4)	0.18	PREDICTED: transitional endoplasmic reticulum ATPase TER94 [Acyrtosiphon pisum]
53	dbj BAH71608.1	84	34130	4 (3)	3 (2)	0.23	ACYPI006696 [Acyrtosiphon pisum]; regucalcin-like [Acyrtosiphon pisum]
54	ref XP_001944221.1	83	89540	12 (2)	10 (2)	0.08	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] [Acyrtosiphon pisum]
55	ref XP_001943110.2	83	96981	10 (2)	8 (2)	0.08	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3 isoform X2 [Acyrtosiphon pisum]
56	ref XP_001945190.2	81	259365	10 (1)	10 (1)	0.01	PREDICTED: fatty acid synthase isoform X1 [Acyrtosiphon pisum]
57	ref XP_008185828.1	81	52687	4 (2)	4 (2)	0.15	PREDICTED: sorting nexin-6 isoform X1 [Acyrtosiphon pisum]
58	ref XP_001952053.1	77	46647	8 (1)	5 (1)	0.08	PREDICTED: eukaryotic initiation factor 4A [Acyrtosiphon pisum]
59	ref XP_001952179.2	75	76309	7 (3)	7 (3)	0.15	PREDICTED: dynamin-1-like protein [Acyrtosiphon pisum]
60	ref XP_008183648.1	74	61191	4 (2)	3 (2)	0.13	PREDICTED: tubulointerstitial nephritis antigen-like [Acyrtosiphon pisum]
61	ref XP_001946553.1	73	46394	3 (1)	3 (1)	0.08	PREDICTED: isocitrate dehydrogenase [NADP] cytoplasmic [Acyrtosiphon pisum]
62	ref XP_001947630.1	72	56070	5 (3)	3 (2)	0.14	PREDICTED: pyruvate kinase isoform X2 [Acyrtosiphon pisum]
63	dbj BAH71451.1	71	26351	2 (1)	2 (1)	0.14	ACYPI002948 [Acyrtosiphon pisum]; uncharacterized protein LOC100161749 [Acyrtosiphon pisum]
64	ref XP_001945383.1	70	28773	1 (1)	1 (1)	0.13	PREDICTED: proteasome subunit alpha type-5 [Acyrtosiphon pisum]
65	ref XP_016659427.1	70	65489	13 (2)	4 (2)	0.12	PREDICTED: threonine--tRNA ligase, cytoplasmic [Acyrtosiphon pisum]
66	ref XP_001948469.2	68	48598	5 (2)	5 (2)	0.16	PREDICTED: protein DDH1 homolog 2 isoform X2 [Acyrtosiphon pisum]
67	ref XP_001945388.2	67	54184	4 (1)	3 (1)	0.07	PREDICTED: uncharacterized protein LOC100160440 [Acyrtosiphon pisum]
68	dbj BAH71339.1	66	26144	3 (1)	3 (1)	0.15	ACYPI006329 [Acyrtosiphon pisum]; ubiquitin C-terminal hydrolase UCHL1-like [Acyrtosiphon pisum]
69	ref XP_001950254.2	64	52670	6 (3)	4 (3)	0.23	PREDICTED: 6-phosphogluconate dehydrogenase, decarboxylating [Acyrtosiphon pisum]
70	ref XP_001951792.1	64	88799	4 (2)	4 (2)	0.09	PREDICTED: heat shock 70 kDa protein 4 [Acyrtosiphon pisum]
71	ref XP_003244666.1	63	55313	2 (1)	2 (1)	0.07	PREDICTED: ubiquitin carboxyl-terminal hydrolase 14 [Acyrtosiphon pisum]
72	dbj BAH70545.1	63	30706	5 (5)	2 (2)	0.26	ACYPI005674 [Acyrtosiphon pisum]; proteasome subunit alpha type-1 [Acyrtosiphon pisum]
73	ref XP_001950393.1	63	43452	3 (1)	2 (1)	0.09	PREDICTED: leukocyte elastase inhibitor [Acyrtosiphon pisum]
74	ref XP_001952191.1	61	97452	11 (4)	6 (3)	0.12	PREDICTED: cytoplasmic aconitate hydratase [Acyrtosiphon pisum]
75	ref XP_016660919.1	60	13423	1 (1)	1 (1)	0.29	PREDICTED: glycogen [starch] synthase-like isoform X1 [Acyrtosiphon pisum]
76	ref XP_016664569.1	60	21062	6 (3)	3 (1)	0.18	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase 1-like [Acyrtosiphon pisum]
77	gb ABD72701.1	59	28868	8 (4)	7 (4)	0.64	putative phosphoglycerate mutase [Acyrtosiphon pisum]
78	ref XP_003245852.1	59	24695	3 (1)	3 (1)	0.15	PREDICTED: tRNA dimethylallyltransferase, mitochondrial [Acyrtosiphon pisum]
79	dbj BAH72584.1	58	17860	5 (2)	3 (1)	0.22	ACYPI007294 [Acyrtosiphon pisum]; uncharacterized protein LOC100166422 precursor [Acyrtosiphon pisum]
80	ref XP_008183142.1	57	53540	3 (1)	3 (1)	0.07	PREDICTED: selenium-binding protein 1-B [Acyrtosiphon pisum]
81	ref XP_008180680.1	56	26335	2 (1)	2 (1)	0.14	PREDICTED: esterase E4 [Acyrtosiphon pisum]
82	ref XP_001950499.3	55	73704	6 (1)	6 (1)	0.05	PREDICTED: stress-70 protein, mitochondrial [Acyrtosiphon pisum]
83	ref XP_001947490.1	55	69899	8 (2)	7 (2)	0.11	PREDICTED: ATP-binding cassette sub-family F member 2 [Acyrtosiphon pisum]

84	ref XP_001952443.1	55	56050	4 (1)	3 (1)	0.07	PREDICTED: importin subunit alpha-4 [Acyrtosiphon pisum]
85	ref XP_001944310.1	55	44527	4 (1)	4 (1)	0.08	PREDICTED: probable phosphoglycerate kinase [Acyrtosiphon pisum]
86	ref XP_001946297.2	55	60404	5 (3)	3 (2)	0.13	PREDICTED: myosinase 1 isoform X1 [Acyrtosiphon pisum]
87	ref XP_001945956.2	55	76068	5 (2)	5 (2)	0.10	PREDICTED: very long-chain-fatty-acid--CoA ligase bubblegum [Acyrtosiphon pisum]
88	ref XP_008179246.1	54	61696	6 (1)	5 (1)	0.06	PREDICTED: apoptosis-inducing factor 3 isoform X1 [Acyrtosiphon pisum]
89	ref XP_001947713.2	53	31982	6 (3)	5 (2)	0.25	PREDICTED: LOW QUALITY PROTEIN: hydroxysteroid dehydrogenase-like protein 2 [Acyrtosiphon pisum]
90	dbj BAH71765.1	53	19613	1 (1)	1 (1)	0.20	ACYPI007317 [Acyrtosiphon pisum]; 15-hydroxyprostaglandin dehydrogenase [NAD+]-like [Acyrtosiphon pisum]
91	dbj BAH70990.1	53	21377	1 (1)	1 (1)	0.18	ACYPI008005 [Acyrtosiphon pisum]; thioredoxin-like protein 1 [Acyrtosiphon pisum]
92	dbj BAH70729.1	48	24327	3 (1)	3 (1)	0.16	ACYPI009884 [Acyrtosiphon pisum]; Thioredoxin
93	dbj BAH72695.1	47	17696	5 (1)	4 (1)	0.22	ACYPI008164 [Acyrtosiphon pisum]
94	dbj BAH71733.1	47	28048	3 (1)	3 (1)	0.13	ACYPI003814 [Acyrtosiphon pisum]
95	dbj BAH71397.1	46	28341	2 (2)	2 (2)	0.29	ACYPI005456 [Acyrtosiphon pisum]; Phosphomannomutase
96	dbj BAH71009.1	46	13421	1 (1)	1 (1)	0.29	ACYPI005557 [Acyrtosiphon pisum]
97	dbj BAH70647.1	46	34445	4 (1)	4 (1)	0.11	ACYPI003593 [Acyrtosiphon pisum]; structural constituent of ribosome
98	dbj BAH71399.1	45	25376	3 (1)	3 (1)	0.15	ACYPI003856 [Acyrtosiphon pisum]; Phosducin-thioredoxin-like
99	ref XP_001948435.1	45	81077	3 (1)	3 (1)	0.05	PREDICTED: influenza virus NS1A-binding protein isoform X1 [Acyrtosiphon pisum]
100	ref XP_008186908.1	45	96467	4 (1)	3 (1)	0.04	PREDICTED: probable glutamine--tRNA ligase isoform X1 [Acyrtosiphon pisum]
101	ref XP_001945112.2	45	99528	5 (1)	5 (1)	0.04	PREDICTED: 26S proteasome non-ATPase regulatory subunit 2 [Acyrtosiphon pisum]
102	ref XP_001947007.1	45	73066	3 (1)	3 (1)	0.05	PREDICTED: glutamate--cysteine ligase catalytic subunit [Acyrtosiphon pisum]
103	gb EFW12069.1	44	8637	2 (1)	2 (1)	0.47	hypothetical protein SSYM_1720 [Serratia symbiotica str. Tucson]
104	ref XP_008179705.1	44	111340	10 (1)	9 (1)	0.03	PREDICTED: uncharacterized protein LOC103308315 [Acyrtosiphon pisum]
105	tpg DAA06105.1	44	38216	1 (1)	1 (1)	0.10	TPA_inf: cathepsin B [Acyrtosiphon pisum]
106	ref XP_001948990.1	43	83295	3 (1)	3 (1)	0.04	PREDICTED: vesicle-fusing ATPase 1 [Acyrtosiphon pisum]
107	ref XP_003244866.1	43	54050	1 (1)	1 (1)	0.07	PREDICTED: signal transducing adapter molecule 1 [Acyrtosiphon pisum]
108	ref XP_001945437.1	43	59127	4 (1)	4 (1)	0.06	PREDICTED: tyrosine--tRNA ligase, cytoplasmic [Acyrtosiphon pisum]
109	ref XP_001947370.2	43	71863	3 (1)	2 (1)	0.05	PREDICTED: beta-galactosidase [Acyrtosiphon pisum]
110	ref XP_001949775.1	42	44356	2 (1)	2 (1)	0.08	PREDICTED: gelsolin-like [Acyrtosiphon pisum]
111	dbj BAH70919.1	41	25229	2 (1)	2 (1)	0.15	ACYPI009786 [Acyrtosiphon pisum]; Chitin-binding type-2 domain protein
112	ref XP_003243194.1	41	74201	9 (1)	8 (1)	0.05	PREDICTED: uncharacterized protein LOC100572209 [Acyrtosiphon pisum]
113	ref XP_016664247.1	41	17616	1 (1)	1 (1)	0.22	PREDICTED: uncharacterized protein LOC107885219 [Acyrtosiphon pisum]
114	ref XP_008181946.1	40	36745	3 (1)	3 (1)	0.10	PREDICTED: ubiquitin carboxyl-terminal hydrolase isozyme L5 [Acyrtosiphon pisum]
115	ref XP_001952329.1	40	61961	4 (1)	4 (1)	0.06	PREDICTED: glucose-6-phosphate isomerase [Acyrtosiphon pisum]
116	dbj BAH71641.1	40	35200	7 (2)	5 (1)	0.11	ACYPI004406 [Acyrtosiphon pisum]; CRAL-TRIO lipid binding domain protein
117	ref XP_008183846.1	40	84248	5 (1)	5 (1)	0.04	PREDICTED: LOW QUALITY PROTEIN: glycine--tRNA ligase [Acyrtosiphon pisum]
118	ref XP_001945199.2	39	77709	6 (1)	6 (1)	0.05	PREDICTED: glutamine--fructose-6-phosphate aminotransferase [isomerizing] 2 isoform X1 [Acyrtosiphon pisum]
119	ref XP_001947487.1	38	60828	6 (1)	3 (1)	0.06	PREDICTED: uncharacterized protein LOC100162406 [Acyrtosiphon pisum]
120	sp C4K744.1 RSMH_HAMD5	38	35313	4 (1)	4 (1)	0.11	RecName: Full=Ribosomal RNA small subunit methyltransferase H; AltName: Full=16S rRNA m(4)C1402 methyltransferase; AltName: Full=rRNA (cytosine-N(4)-)-methyltransferase RsmH
121	dbj BAH71722.1	38	37742	2 (1)	2 (1)	0.10	ACYPI001175 [Acyrtosiphon pisum]; Papain-like cysteine peptidase
122	ref XP_001951757.1	37	63972	7 (1)	6 (1)	0.06	PREDICTED: asparagine--tRNA ligase, cytoplasmic [Acyrtosiphon pisum]
123	ref XP_001943357.1	36	103383	11 (1)	8 (1)	0.04	PREDICTED: staphylococcal nuclease domain-containing protein 1 [Acyrtosiphon pisum]
124	gb EFW11981.1	36	74782	2 (1)	2 (1)	0.05	putative relaxase/mobilization protein [Serratia symbiotica str. Tucson]
125	dbj BAH70594.1	36	35782	9 (2)	6 (2)	0.22	ACYPI006664 [Acyrtosiphon pisum]; D-isomer specific 2-hydroxyacid dehydrogenase, catalytic domain

126	dbj BAH72929.1	36	20943	4 (2)	3 (2)	0.40	ACYPI005249 [Acyrtosiphon pisum]
127	ref XP_008182947.1	35	230711	21 (3)	15 (1)	0.02	PREDICTED: uncharacterized protein LOC103307628 [Acyrtosiphon pisum]
128	ref XP_001944313.3	35	79842	4 (1)	3 (1)	0.05	PREDICTED: 1,4-alpha-glucan-branching enzyme [Acyrtosiphon pisum]
129	ref XP_001950987.1	35	35527	5 (1)	4 (1)	0.11	PREDICTED: 26S proteasome non-ATPase regulatory subunit 7 [Acyrtosiphon pisum]
130	dbj BAH72892.1	34	30304	6 (1)	5 (1)	0.12	ACYPI010127 [Acyrtosiphon pisum]
131	ref XP_001943774.1	34	36505	1 (1)	1 (1)	0.10	PREDICTED: serine/threonine-protein phosphatase 4 catalytic subunit isoform X1 [Acyrtosiphon pisum]
132	ref XP_003241972.1	34	101920	4 (1)	4 (1)	0.04	PREDICTED: uncharacterized protein LOC100569797 [Acyrtosiphon pisum]
133	ref XP_001951748.2	34	58747	3 (1)	3 (1)	0.06	PREDICTED: importin subunit alpha-7 [Acyrtosiphon pisum]
134	dbj BAH72639.1	34	29027	1 (1)	1 (1)	0.13	ACYPI46563 [Acyrtosiphon pisum]
135	dbj BAH72940.1	33	20297	6 (1)	5 (1)	0.19	ACYPI003483 [Acyrtosiphon pisum]
136	ref XP_001950269.1	33	13806	5 (1)	3 (1)	0.29	PREDICTED: histone H2B-like [Acyrtosiphon pisum]
137	ref XP_001951813.1	33	100283	2 (1)	2 (1)	0.04	PREDICTED: AMP deaminase 2 isoform X5 [Acyrtosiphon pisum]
138	ref XP_001950205.1	33	42811	2 (2)	2 (2)	0.18	PREDICTED: leukocyte elastase inhibitor [Acyrtosiphon pisum]
139	ref XP_008184045.1	32	191388	12 (1)	10 (1)	0.02	PREDICTED: kinase D-interacting substrate of 220 kDa isoform X2 [Acyrtosiphon pisum]
140	ref XP_008178697.1	32	83020	5 (1)	4 (1)	0.04	PREDICTED: E3 SUMO-protein ligase KIAA1586-like [Acyrtosiphon pisum]
141	ref XP_008187316.1	32	31978	1 (1)	1 (1)	0.12	PREDICTED: acidic leucine-rich nuclear phosphoprotein 32 family member A-like [Acyrtosiphon pisum]
142	ref XP_001948977.1	32	60626	7 (1)	7 (1)	0.06	PREDICTED: T-complex protein 1 subunit gamma-like [Acyrtosiphon pisum]
143	ref XP_008178955.1	31	43547	3 (1)	3 (1)	0.09	PREDICTED: serpin B4-like isoform X1 [Acyrtosiphon pisum]
144	ref XP_001948873.1	31	119754	5 (1)	5 (1)	0.03	PREDICTED: flocculation protein FLO11 [Acyrtosiphon pisum]
145	ref XP_003240648.1	31	67316	7 (1)	5 (1)	0.06	PREDICTED: cytosolic purine 5'-nucleotidase isoform X3 [Acyrtosiphon pisum]
146	ref XP_008187164.1	30	114951	6 (1)	6 (1)	0.03	PREDICTED: lysosomal alpha-mannosidase isoform X1 [Acyrtosiphon pisum]
147	ref XP_016665172.1	30	40077	2 (1)	2 (1)	0.09	PREDICTED: uncharacterized serine-rich protein C215.13-like [Acyrtosiphon pisum]
148	dbj BAH72452.1	30	26442	5 (1)	5 (1)	0.14	ACYPI001319 [Acyrtosiphon pisum]; COP9 signalosome complex subunit 7 domain
149	gb EFW12911.1	30	26896	4 (1)	3 (1)	0.14	broad specificity 5'(3')-nucleotidase and polyphosphatase [Serratia symbiotica str. Tucson]
150	ref XP_016664520.1	30	36383	1 (1)	1 (1)	0.10	PREDICTED: DNA ligase B-like, partial [Acyrtosiphon pisum]
151	dbj BAH72598.1	29	34260	3 (1)	2 (1)	0.11	ACYPI004131 [Acyrtosiphon pisum]
152	gb EFW11712.1	28	90869	3 (1)	2 (1)	0.04	putative truncated conjugative transfer ATPase, PFL_4706 family [Serratia symbiotica str. Tucson]
153	ref XP_008182063.2	28	104474	3 (1)	3 (1)	0.04	PREDICTED: (E3-independent) E2 ubiquitin-conjugating enzyme UBE20-like [Acyrtosiphon pisum]
154	ref XP_008187287.1	28	90498	4 (1)	3 (1)	0.04	PREDICTED: uncharacterized WD repeat-containing protein C1306.02-like [Acyrtosiphon pisum]
155	dbj BAH70974.1	28	20606	12 (1)	7 (1)	0.19	ACYPI009103 [Acyrtosiphon pisum]
156	sp B8D6Z9.1 TAL_BUCAT	27	35542	2 (1)	2 (1)	0.11	RecName: Full=Transaldolase
157	ref XP_001951085.1	26	95184	3 (1)	3 (1)	0.04	PREDICTED: importin subunit beta [Acyrtosiphon pisum]
158	ref XP_008189727.1	26	26357	1 (1)	1 (1)	0.14	PREDICTED: uncharacterized protein LOC103311793, partial [Acyrtosiphon pisum]
159	ref XP_001943896.1	25	91598	4 (1)	4 (1)	0.04	PREDICTED: vacuolar protein sorting-associated protein 16 homolog [Acyrtosiphon pisum]
160	ref XP_001944905.2	24	224118	14 (1)	11 (1)	0.02	PREDICTED: autophagy-related protein 2 homolog B [Acyrtosiphon pisum]
161	ref XP_008187300.1	23	137024	5 (1)	5 (1)	0.03	PREDICTED: mucin-5AC isoform X2 [Acyrtosiphon pisum]
162	gb KHG03609.1	22	132057	6 (1)	5 (1)	0.03	Acetolactate synthase large subunit [Gossypium arboreum]
163	ref XP_016664427.1	21	58827	3 (1)	2 (1)	0.06	PREDICTED: uncharacterized protein LOC107885320 [Acyrtosiphon pisum]
164	sp B8D740.1 SYGB_BUCAT	19	80808	15 (1)	14 (1)	0.05	RecName: Full=Glycine-tRNA ligase beta subunit; AltName: Full=Glycyl-tRNA synthetase beta subunit; Short=GlyRS
165	ref XP_008181787.1	19	18216	3 (1)	2 (1)	0.21	PREDICTED: uncharacterized protein LOC103309049 [Acyrtosiphon pisum]
166	ref XP_001948177.2	17	71393	7 (1)	7 (1)	0.05	PREDICTED: voltage-dependent L-type calcium channel subunit beta-1 [Acyrtosiphon pisum]
167	dbj BAB13053.1	15	36591	5 (1)	4 (1)	0.10	ribosomal large subunit pseudouridine synthase C [Buchnera aphidicola str. APS (Acyrtosiphon pisum)]

Supplementary figures

MADKNNILYLFDRPTEPIFIGKGEENVSFVDPDYLIDRYKPLASDIQTRFPGGKTPVPT	60
M DKNNILYLFDRPTEPIFIGK++NVSF+VP +YL DRYKPLASDIQ RF GKT+ VT	
MTDKNNILYLFDRPTEPIFIGKDDNVSFEVPAEYLTDRYKPLASDIQNRFSGKTISVT	60
RLSSIPDLSIPLGLKRDMPFSLFNQSHGKMAAKLIEILMNAKSYDELLSLSVYCRDRINP	120
+LSSIPD+S PL L RD FSLF H KMAAKLIEI M +K++DELLSL+VY RDR+NP	
KLSSIPDMSFPLQLGRDKAFSLFIPYHSKMAAKLIEIFMASKTFDELLSLAVYARDRVNP	120
YMFTYALSVALIHRPDTRNLRPSHSEMFPPLYMDSSV FARAREESAVV QTGSRTPIEIP	180
YMF YALSV + HRPDTRNL LPSH EMFPPLYMD++VF RAREESAVV QTGSRTPIEIP	
YMFIYALSVVVTTHRPDTRNLELPSHVEMFPPLYMDATVFG RAREESAVV QTGSRTPIEIP	180
HDY SANNLDAEHRISYFREDIGINLHHHWHHLVYPFDGPLNIVNKNRRGELFFYMHQQII	240
HDY SAN+LD EHRISYFREDIG+NLHHHWHHLVYPFDGP++IVNK+RRGELF+YMH+QI+	
HDY SANDLDFEHRISYFREDIGVNLHHHWHHLVYPFDGPVDIVNKDRRGELFFYMHEQIL	240
ARYNMERLSNMMNRVRLTNWDQPIAEGYFPK LDNILANR VWPPRPVNAV LQNISREVEQ	300
ARYNMERLSN+MNRVRLTNW PI EGYFPK LDNILANR VWP RPVNA L NI+RE+EQ	
ARYNMERLSNDMMNRVRLTNWRSPILEGYFPK LDNILANR VWPSRPVNATLSNINREIEQ	300
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I+FDIEDL RWRDRIFNAIHSGFIINTAGQQVRLTE DGI+ILGN+IE+SILS N NLYG	
ISFDIEDLERWRDRIFNAIHSGFIINTAGQQVRLTEADGINILGNLIEASILSLNQNLYG	360
SLHNNGHNAIAYIHDPNRFLFENYSVMGDSATAMR DPIFYR WHAYIDDIFQEYKATIPSY	420
SLHNNGHNAI++IHDPNRFLFENY VMGDSATAMR DPIFYR WHAYIDDIFQE+KATIPSY	
SLHNNGHNAISFIHDPNRFLFENYVMGDSATAMR DPIFYR WHAYIDDIFQEFKATIPSY	420
NVQNLGFDNVSQSVQVEVTATGLPRNEFATFWQSDTDLR GLDFLPRGSV FARFTHLQHA	480
+QNL FDNV VQSVQVE++ATG+PRNE ATFWQSD DLSR GLDFLPRGSV FARFTHLQHA	
TIQNLSFDNVRVQSVQVEISATGIPRNELATFWQSDVDLSR GLDFLPRGSV FARFTHLQHA	480
PFNYKI IVENNGNQ RIGTVRIFLAPKFDER GLPFLFREQR KLFVELDKFSTSLKRGRNEI	540
PFNYKI VENNGNQ+GTVRIF+APK+DER GLPFLFREQR KL VELDKFS +L RGRNEI	
PFNYKITVENNGNQRLGTVRIFIAPKYDER GLPFLFREQR KLMVELDKFSVTLTRGRNEI	540
VRSIESSVTIPHEITYRNQGSNRPAANSDAAPMFNFCGCGWPQNMLIAKGSPEGFQCQL	600
RRSIESSVTIPHEITYRN NRPA NSDAA FNFCGCGWPQNMLI KG+ EGFQCQL	
TRRSIESSVTIPHEITYRNLDNRNPANNSDAAAANFCGCGWPQNMLIPKGTAEGFQCQL	600
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FVM+SNG DQV NAQ DGQTCDD+ASSYCGI NSRYPD+R SMGYPFDRTPRDG VVTLQQF	
FVMISNGANDQVENAQAQGDGQTCDDNASSYCGIRNSRYPDAR SMGYPFDRTPRDG VVTLQQF	660
LTNMVVQDVRI RFSNRT VAPLQ NATANRNAGTSNNNKRN 701	
LT NMVVQDVRI RFSNRT VAPLQ N K	
LTPNMVVQDVRI RFSNRT VAPLQ NRIGSQQTSKNPPAKAPGRN 704	

Figure S1. Comparisons of the two pea aphid PO sequences. Top sequence: XP_001951137.1, phenoloxidase 2 (PO2-X1); bottom sequence: XP_001949307.1 phenoloxidase 2 (PO2); middle sequence: identical amino acid or conservative replacement (+). In red: peptides used for immunization; In green: peptides identified by mass spectrometry in spot 6 (Fig.1).

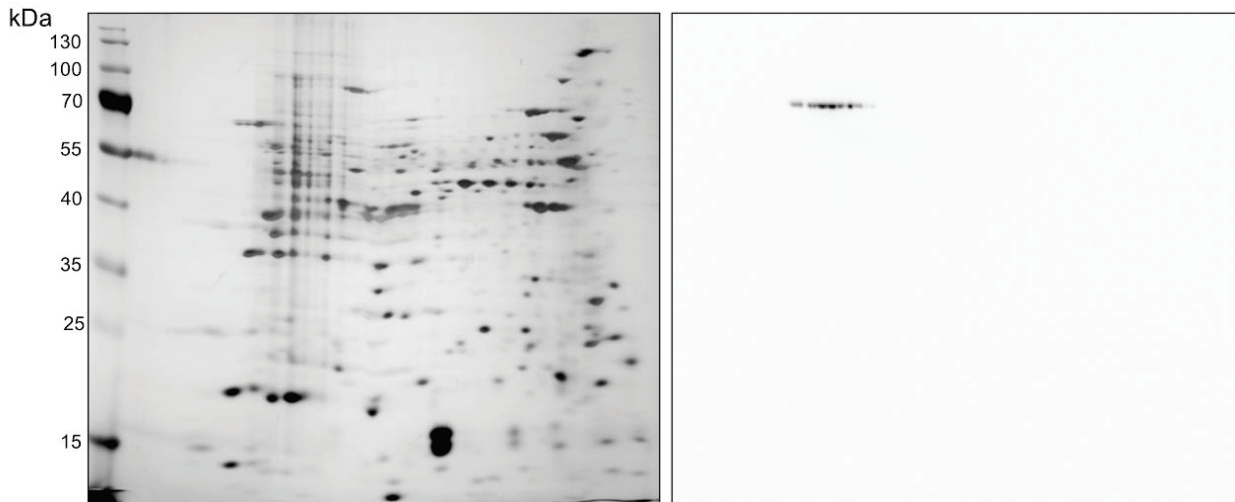


Figure S2. Anti-PO antibody test on 2D gel of LL01 hemolymph. LL01 hemolymph 2D silver stained gel (left; from Figure 1) and the equivalent western blot obtained after transfer of the same amount of proteins and probed with the rabbit polyclonal anti-PO peptides (right). SDS-PAGE, 12,5%; Molecular weight in kDa. Left, Ac side; right basic side.

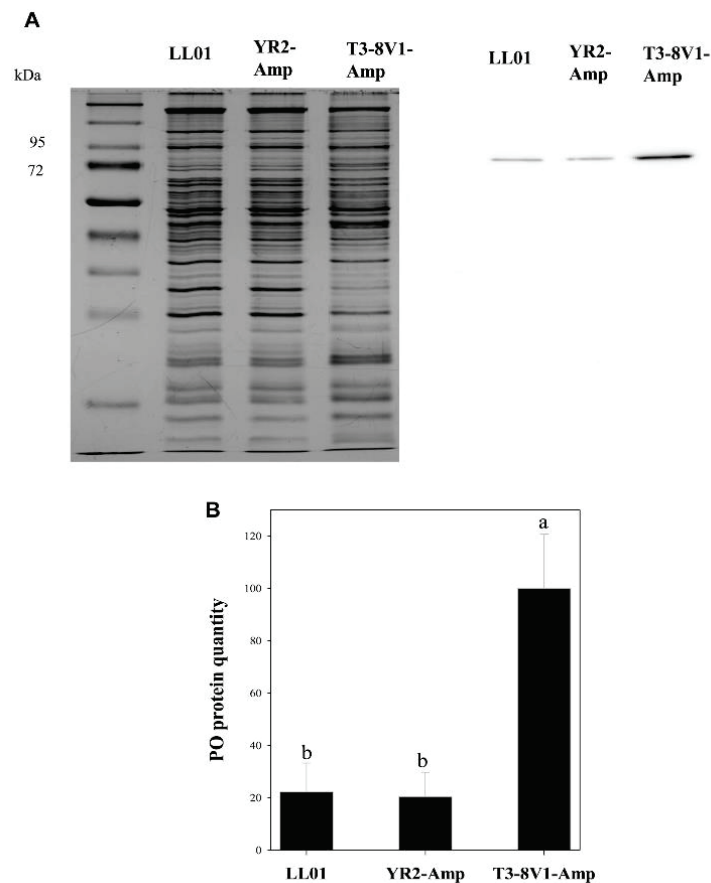


Figure S3. Comparison of hemolymph PO protein quantity between the three aphid genetic backgrounds, LL01, YR2-Amp and T3-8V1-amp. (A) the silver stained gel showing the hemolymph protein quantity loaded (left) and the equivalent western blot showing the differences in the PO protein signal (right). (B) PO protein quantity calculated by dividing the PO western blot signal by the total amount of protein (see mat. and meth.) (for simplification the highest value for T3-8V1-amp was fixed at 100%). Results expressed as mean \pm SEM and different letters (a, b) show significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 3$. Molecular weight in kDa.

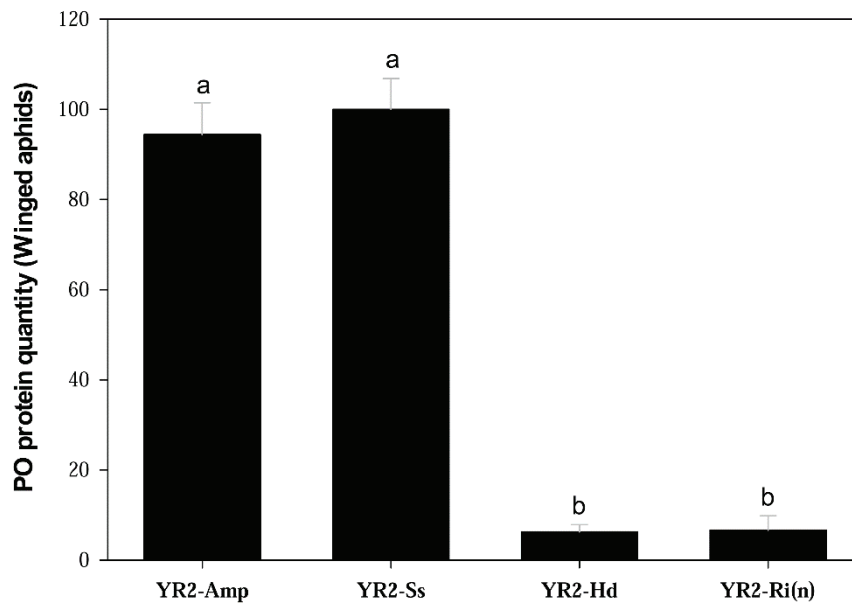


Figure S4. Comparison of hemolymph PO protein quantity in alate aphids from the different YR2 lines. Alate aphid hemolymph PO protein quantity was calculated by dividing the PO signal from western blot by the total amount of protein (see mat. and meth.) (for simplification the highest value obtained for YR2-Ss was fixed at 100%). Results expressed as mean \pm SEM and different letters (a, b) show significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 3$.

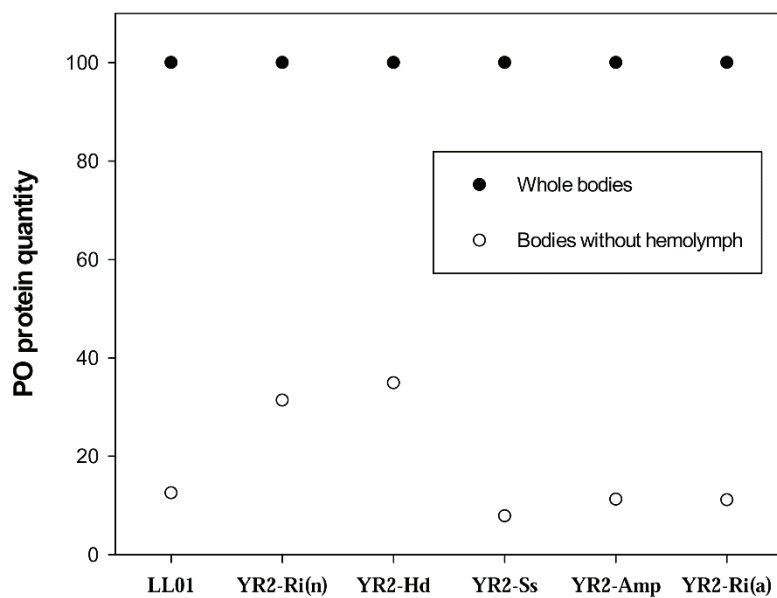


Figure S5. Proportion of PO protein remaining in the bled aphid carcasses. To estimate the hemolymph circulating PO quantity in each line, PO protein quantity was measured in the whole aphids (value fixed at 100% for each line) and in the aphids after bleeding (bodies without hemolymph) for the different indicated lines, $n = 3$.

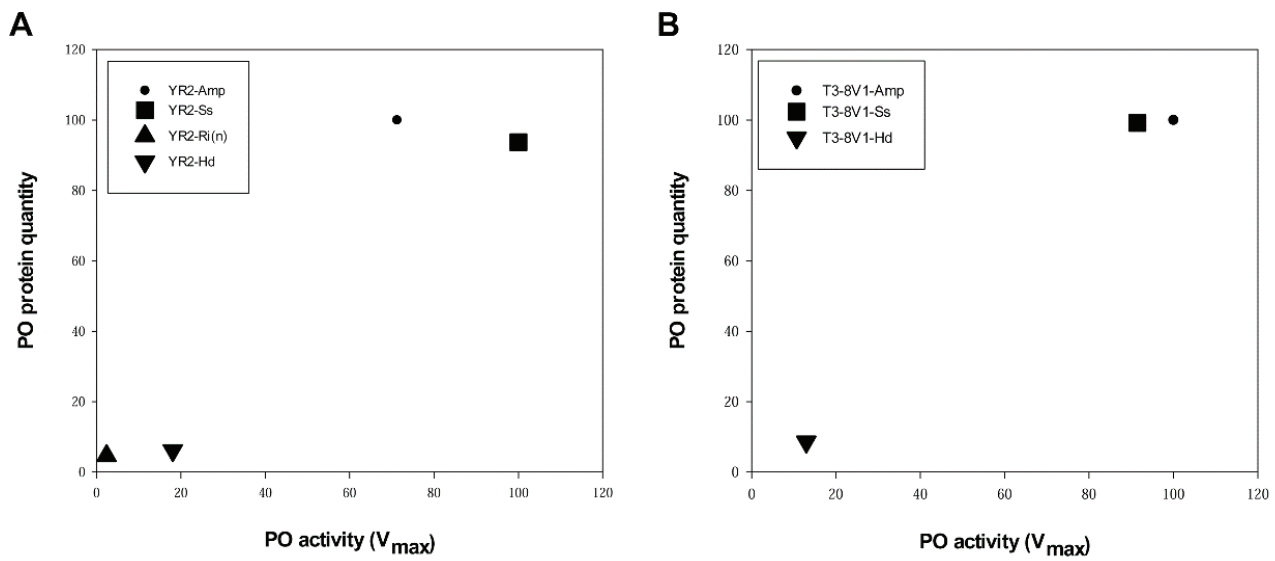


Figure S6. Correlation between PO hemolymph quantity and activity. Correlation between PO hemolymph quantity and activity (A) among different YR2 and (B) among T3-8V1 aphid lines. (PO quantity for YR2 and T3-8V1 lines from Fig 3B and Fig 3D, respectively, $n = 3$ for PO protein quantity and $n > 6$ for PO activity).

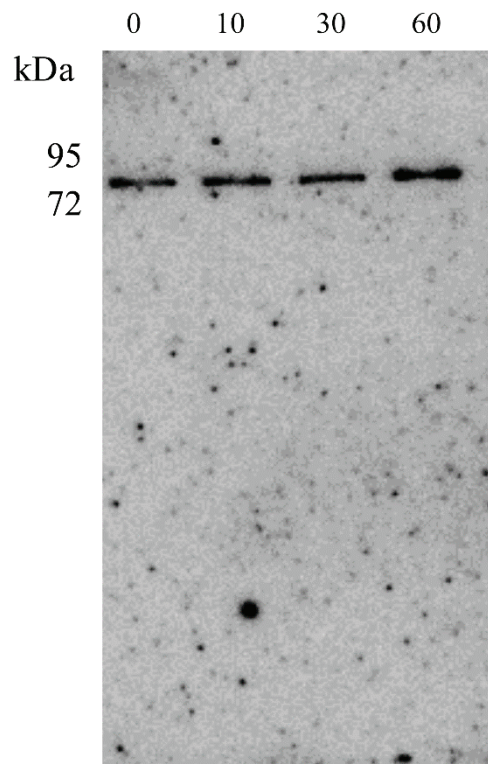


Figure S7. Absence of cleavage activation of aphid PO for activity. Hemolymph from YR2-amp was collected in water (as for the activity assay, see mat. and meth.) and left at room temperature for one hour. Aliquots were collected at 0, 10, 30 and 60 min, separated on a 12,5% SDS-PAGE in reducing conditions and proteins transferred to nitrocellulose. The blot probed with the anti-PO antibody showed only one reactive band at about 80 kDa in all samples. Similar results were obtained with LL01 and T3-8V1-amp (not shown). MW in kDa.

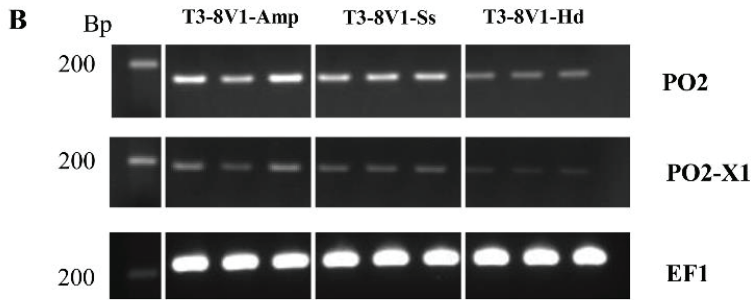
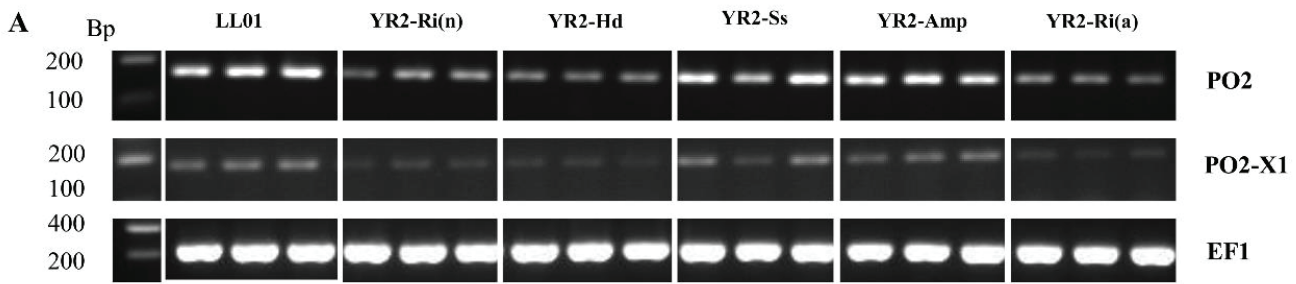


Figure S8. Agarose gels of RT-PCR products for PO2, PO2-X1 and EF1. Pictures of the PCR products for PO2, PO2-X1 and EF1 for LL01 and YR2 aphid lines (A) and for T3-8V1 lines (B) separated on a 1.5% agarose gels and stained with ethidium bromide. For each line the 3 products come from three different experiments.

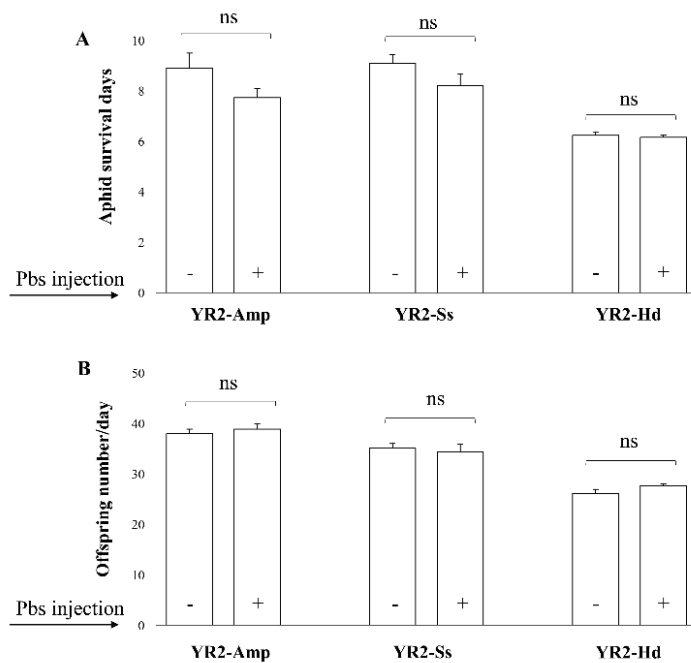


Figure S9. Absence of PBS microinjection effect on life-history traits. The mean aphid survival days (A) and the number of offspring produced per day (B) were compared between un-treated (- columns) and after PBS microinjection (+ columns) for the YR2-amp, YR2-Ss and YR2-Hd lines. Results expressed as mean \pm SEM, n.s., no significant differences (multi-comparison test from “multcomp” package), $n = 3$.

Supplementary File 1

Statistics:

For Figure 3b, 4ab. Results of multi-comparisons analyzing among different aphid lines (LL01 and YR2 genotypes); the ANOVA of whole experiment were $F_{4,15} = 49.759, P < 0.001$; $F_{5,18} = 20.292, P < 0.001$; $F_{5,18} = 37.592, P < 0.001$; $F_{3,16} = 37.054, P < 0.001$ for PO protein, PO2 gene, PO2-X1 gene and PO activity, respectively.

Multi-comparisons	PO protein	PO2 gene	PO2-X1 gene	PO activity
	P-value			
LL01- YR2-Ri(n)	<0.001	0.001	<0.001	
LL01- YR2-Hd	<0.001	<0.001	<0.001	
LL01- YR2-Ss	0.968	0.962	0.723	
LL01- YR2-Amp	0.898	0.993	0.801	
LL01- YR2-Ri(a)		<0.001	<0.001	
YR2-Ri(n)- YR2-Hd	0.947	0.990	0.990	0.573
YR2- Ri(n)- YR2-Ss	<0.001	0.004	<0.001	<0.001
YR2-Ri(n)- YR2-Amp	<0.001	0.002	<0.001	<0.001
YR2-Ri(n)- YR2-Ri(a)		0.920	0.152	
YR2-Hd- YR2-Ss	<0.001	0.001	<0.001	<0.001
YR2-Hd- YR2-Amp	<0.001	<0.001	<0.001	<0.001
YR2-Hd- YR2-Ri(a)		0.999	0.352	
YR2-Ss- YR2-Amp	0.998	0.999	1.000	0.812
YR2-Ss- YR2-Ri(a)		<0.001	<0.001	
YR2-Amp- YR2-Ri(a)		<0.001	<0.001	

For Figure 3d, 4cd, S6. Results of multi-comparisons analyzing among different aphid lines (T3-8V1 genotype); the ANOVA of whole experiment were $F_{2,6} = 346.301, P < 0.001$; $F_{2,6} = 23.320, P = 0.001$; $F_{2,6} = 27.863, P = 0.001$; $F_{2,15} = 172.697, P < 0.001$ for PO protein, PO2 gene, PO2-X1 gene and PO activity, respectively.

Multi-comparisons	PO protein	PO2 gene	PO2-X1 gene	PO activity
	P-value			
T3-8V1-Amp- T3-8V1-Ss	0.985	0.492	0.431	0.607
T3-8V1-Amp- T3-8V1-Hd	<0.001	0.002	0.001	<0.001
T3-8V1-Ss- T3-8V1-Hd	<0.001	0.005	0.003	<0.001

For Figure 5, 6, 7. Results of multi-comparisons analyzing among different aphid lines under different stressors; the statistical results of whole experiment were $F_{15,32} = 43.970, P < 0.001$; $F_{15,85} = 58.311, P < 0.001$; $F_{15,32} = 32.897, P < 0.001$; $F_{15,32} = 28.212, P < 0.001$; $F_{15,130} = 15.600, P < 0.001$; $\chi^2 = 430.860, P < 0.001$ for PO protein, PO activity, PO2 gene and PO2-X1 gene expression as well as aphid offspring/day/aphid pool and survival, respectively.

Multi-comparisons	PO protein	PO activity	PO2 gene	PO2-X1 gene	Offspring	Survival
	P-value					
YR2_Amp_Ecoli - YR2_Amp_beads	0.300	1.000	<0.001	0.801	1.000	0.073
YR2_Amp_Pbs - YR2_Amp_beads	0.999	0.999	<0.001	0.993	0.954	<0.01
YR2_Amp_Yeast - YR2_Amp_beads	<0.001	<0.01	1.000	1.000	1.000	<0.001
YR2_Hd_beads - YR2_Amp_beads	<0.001	<0.001	0.993	1.000	<0.001	0.013
YR2_Hd_Ecoli - YR2_Amp_beads	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001
YR2_Hd_Pbs - YR2_Amp_beads	<0.01	<0.001	1.000	1.000	<0.001	1.000
YR2_Hd_Yeast - YR2_Amp_beads	<0.01	<0.001	0.964	0.998	<0.001	0.171
YR2_Ri(n)_beads - YR2_Amp_beads	0.610	<0.001	0.939	1.000	<0.001	<0.01
YR2_Ri(n)_Ecoli - YR2_Amp_beads	<0.01	<0.001	0.999	1.000	<0.001	<0.001
YR2_Ri(n)_Pbs - YR2_Amp_beads	<0.01	<0.001	<0.001	0.457	<0.001	0.999
YR2_Ri(n)_Yeast - YR2_Amp_beads	0.257	<0.001	0.999	1.000	<0.001	0.089
YR2_Ss_beads - YR2_Amp_beads	1.000	<0.01	0.176	0.211	0.217	0.616
YR2_Ss_Ecoli - YR2_Amp_beads	0.994	<0.01	0.081	0.022	0.803	<0.001
YR2_Ss_Pbs - YR2_Amp_beads	1.000	0.038	<0.001	<0.001	<0.01	<0.001
YR2_Ss_Yeast - YR2_Amp_beads	<0.001	<0.001	0.956	0.884	0.295	0.317
YR2_Amp_Pbs - YR2_Amp_Ecoli	0.051	0.955	1.000	1.000	0.953	<0.001

YR2_Amp_Yeast - YR2_Amp_Ecoli	<0.001	0.051	<0.001	0.814	1.000	<0.001
YR2_Hd_beads - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.966	<0.001	1.000
YR2_Hd_Ecoli - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.813	<0.001	0.702
YR2_Hd_Pbs - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.995	<0.001	0.018
YR2_Hd_Yeast - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.999	<0.001	1.000
YR2_Ri(n)_beads - YR2_Amp_Ecoli	<0.01	<0.001	<0.001	0.839	<0.001	1.000
YR2_Ri(n)_Ecoli - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.343	<0.001	<0.001
YR2_Ri(n)_Pbs - YR2_Amp_Ecoli	<0.001	<0.001	0.190	1.000	<0.001	<0.001
YR2_Ri(n)_Yeast - YR2_Amp_Ecoli	<0.001	<0.001	<0.001	0.668	<0.001	1.000
YR2_Ss_beads - YR2_Amp_Ecoli	0.508	0.035	0.014	0.999	0.215	<0.001
YR2_Ss_Ecoli - YR2_Amp_Ecoli	0.967	0.024	0.038	0.787	0.801	0.187
YR2_Ss_Pbs - YR2_Amp_Ecoli	0.639	0.250	<0.01	<0.001	<0.01	<0.001
YR2_Ss_Yeast - YR2_Amp_Ecoli	0.517	<0.001	<0.001	1.000	0.292	<0.001
YR2_Amp_Yeast - YR2_Amp_Pbs	<0.001	<0.001	<0.001	0.994	0.965	1.000
YR2_Hd_beads - YR2_Amp_Pbs	<0.01	<0.001	<0.001	0.999	0.027	<0.001
YR2_Hd_Ecoli - YR2_Amp_Pbs	0.020	<0.001	0.869	<0.001	<0.001	<0.001
YR2_Hd_Pbs - YR2_Amp_Pbs	0.046	<0.001	<0.001	1.000	<0.001	0.073
YR2_Hd_Yeast - YR2_Amp_Pbs	0.045	<0.001	<0.001	1.000	<0.001	<0.001
YR2_Ri(n)_beads - YR2_Amp_Pbs	0.979	<0.001	<0.001	0.996	<0.01	<0.001
YR2_Ri(n)_Ecoli - YR2_Amp_Pbs	0.015	<0.001	<0.001	0.779	<0.01	<0.001
YR2_Ri(n)_Pbs - YR2_Amp_Pbs	<0.01	<0.001	0.236	0.995	<0.001	0.158
YR2_Ri(n)_Yeast - YR2_Amp_Pbs	0.771	<0.001	<0.001	0.969	0.343	<0.001
YR2_Ss_beads - YR2_Amp_Pbs	0.997	<0.001	0.011	0.921	0.999	0.952
YR2_Ss_Ecoli - YR2_Amp_Pbs	0.728	<0.001	0.028	0.351	1.000	<0.001
YR2_Ss_Pbs - YR2_Amp_Pbs	0.985	<0.01	<0.01	<0.001	0.422	0.987
YR2_Ss_Yeast - YR2_Amp_Pbs	<0.001	<0.01	<0.001	1.000	0.999	0.997
YR2_Hd_beads - YR2_Amp_Yeast	<0.001	<0.001	0.996	1.000	<0.001	<0.001
YR2_Hd_Ecoli - YR2_Amp_Yeast	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
YR2_Hd_Pbs - YR2_Amp_Yeast	<0.001	<0.001	1.000	1.000	<0.001	<0.01
YR2_Hd_Yeast - YR2_Amp_Yeast	<0.001	<0.001	0.977	0.998	<0.001	<0.001
YR2_Ri(n)_beads - YR2_Amp_Yeast	<0.001	<0.001	0.959	1.000	<0.001	<0.001
YR2_Ri(n)_Ecoli - YR2_Amp_Yeast	<0.001	<0.001	0.999	1.000	<0.001	<0.001
YR2_Ri(n)_Pbs - YR2_Amp_Yeast	<0.001	<0.001	<0.001	0.463	<0.001	0.021
YR2_Ri(n)_Yeast - YR2_Amp_Yeast	<0.001	<0.001	0.999	1.000	<0.001	<0.001
YR2_Ss_beads - YR2_Amp_Yeast	<0.001	1.000	0.210	0.215	0.283	0.583
YR2_Ss_Ecoli - YR2_Amp_Yeast	<0.001	1.000	0.097	0.022	0.848	<0.001
YR2_Ss_Pbs - YR2_Amp_Yeast	<0.001	1.000	<0.001	<0.001	<0.01	1.000
YR2_Ss_Yeast - YR2_Amp_Yeast	<0.001	0.190	0.971	0.887	0.367	0.852
YR2_Hd_Ecoli - YR2_Hd_beads	1.000	1.000	<0.001	<0.001	0.858	0.991
YR2_Hd_Pbs - YR2_Hd_beads	1.000	1.000	1.000	1.000	0.524	<0.01
YR2_Hd_Yeast - YR2_Hd_beads	1.000	1.000	1.000	1.000	0.997	1.000
YR2_Ri(n)_beads - YR2_Hd_beads	0.233	0.988	1.000	1.000	1.000	1.000
YR2_Ri(n)_Ecoli - YR2_Hd_beads	1.000	0.803	1.000	0.997	1.000	<0.001
YR2_Ri(n)_Pbs - YR2_Hd_beads	1.000	0.697	<0.001	0.745	0.738	<0.001
YR2_Ri(n)_Yeast - YR2_Hd_beads	0.573	0.990	1.000	1.000	0.998	1.000
YR2_Ss_beads - YR2_Hd_beads	<0.001	<0.001	0.895	0.442	0.362	<0.001
YR2_Ss_Ecoli - YR2_Hd_beads	<0.001	<0.001	0.698	0.065	0.031	0.777
YR2_Ss_Pbs - YR2_Hd_beads	<0.001	<0.001	<0.001	<0.001	0.994	<0.001
YR2_Ss_Yeast - YR2_Hd_beads	<0.001	<0.001	1.000	0.987	0.278	<0.001
YR2_Hd_Pbs - YR2_Hd_Ecoli	1.000	1.000	<0.001	<0.001	1.000	<0.001
YR2_Hd_Yeast - YR2_Hd_Ecoli	1.000	1.000	<0.001	<0.001	1.000	0.701
YR2_Ri(n)_beads - YR2_Hd_Ecoli	0.415	0.998	<0.001	<0.001	0.991	0.970
YR2_Ri(n)_Ecoli - YR2_Hd_Ecoli	1.000	0.916	<0.001	<0.001	0.861	<0.001
YR2_Ri(n)_Pbs - YR2_Hd_Ecoli	1.000	0.845	0.998	<0.001	1.000	<0.001
YR2_Ri(n)_Yeast - YR2_Hd_Ecoli	0.793	0.999	<0.001	<0.001	0.100	0.659
YR2_Ss_beads - YR2_Hd_Ecoli	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
YR2_Ss_Ecoli - YR2_Hd_Ecoli	<0.001	<0.001	<0.001	0.046	<0.001	1.000
YR2_Ss_Pbs - YR2_Hd_Ecoli	<0.001	<0.001	0.255	<0.001	0.073	<0.001
YR2_Ss_Yeast - YR2_Hd_Ecoli	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
YR2_Hd_Yeast - YR2_Hd_Pbs	1.000	1.000	1.000	1.000	0.998	0.051
YR2_RiN_beads - YR2_Hd_Pbs	0.646	0.986	0.999	1.000	0.856	<0.01
YR2_Ri(n)_Ecoli - YR2_Hd_Pbs	1.000	0.789	1.000	0.976	0.505	<0.001
YR2_Ri(n)_Pbs - YR2_Hd_Pbs	0.999	0.681	<0.001	0.889	1.000	1.000
YR2_Ri(n)_Yeast - YR2_Hd_Pbs	0.940	0.989	1.000	0.999	0.020	0.021
YR2_Ss_beads - YR2_Hd_Pbs	<0.01	<0.001	0.483	0.628	<0.001	0.945
YR2_Ss_Ecoli - YR2_Hd_Pbs	<0.001	<0.001	0.271	0.123	<0.001	<0.001
YR2_Ss_Pbs - YR2_Hd_Pbs	<0.01	<0.001	<0.001	<0.001	0.014	<0.001
YR2_Ss_Yeast - YR2_Hd_Pbs	<0.001	<0.001	0.999	0.999	<0.01	0.762
YR2_RiN_beads - YR2_Hd_Yeast	0.635	0.980	1.000	0.999	1.000	1.000
YR2_RiN_Ecoli - YR2_Hd_Yeast	1.000	0.756	1.000	0.857	0.998	<0.001
YR2_RiN_Pbs - YR2_Hd_Yeast	0.999	0.643	<0.001	0.985	1.000	<0.01
YR2_RiN_Yeast - YR2_Hd_Yeast	0.936	0.984	1.000	0.988	0.464	1.000
YR2_Ss_beads - YR2_Hd_Yeast	<0.01	<0.001	0.965	0.864	0.011	<0.001
YR2_Ss_Ecoli - YR2_Hd_Yeast	<0.001	<0.001	0.841	0.272	<0.001	0.208
YR2_Ss_Pbs - YR2_Hd_Yeast	<0.01	<0.001	<0.001	<0.001	0.383	<0.001
YR2_Ss_Yeast - YR2_Hd_Yeast	<0.001	<0.001	1.000	1.000	<0.01	<0.001

YR2_Ri(n)_Ecoli - YR2_Ri(n)_beads	0.350	1.000	1.000	1.000	1.000	<0.001
YR2_Ri(n)_Pbs - YR2_Ri(n)_beads	0.113	1.000	<0.001	0.494	0.968	<0.001
YR2_Ri(n)_Yeast - YR2_Ri(n)_beads	1.000	1.000	1.000	1.000	0.840	1.000
YR2_Ss_beads - YR2_Ri(n)_beads	0.385	<0.001	0.981	0.236	0.052	<0.001
YR2_Ss_Ecoli - YR2_Ri(n)_beads	0.064	<0.001	0.889	0.026	<0.01	0.606
YR2_Ss_Pbs - YR2_Ri(n)_beads	0.278	<0.001	<0.001	<0.001	0.768	<0.001
YR2_Ss_Yeast - YR2_Ri(n)_beads	<0.001	<0.001	1.000	0.906	0.033	<0.001
YR2_RiN_Pbs - YR2_Ri(n)_Ecoli	1.000	1.000	<0.001	0.121	0.730	<0.001
YR2_RiN_Yeast - YR2_Ri(n)_Ecoli	0.728	1.000	1.000	1.000	0.990	<0.001
YR2_Ss_beads - YR2_Ri(n)_Ecoli	<0.001	<0.001	0.745	0.041	0.212	<0.001
YR2_Ss_Ecoli - YR2_Ri(n)_Ecoli	<0.001	<0.001	0.502	<0.01	0.010	<0.01
YR2_Ss_Pbs - YR2_Ri(n)_Ecoli	<0.001	<0.001	<0.001	<0.001	0.977	<0.001
YR2_Ss_Yeast - YR2_Ri(n)_Ecoli	<0.001	<0.001	1.000	0.436	0.152	<0.001
YR2_RiN_Yeast - YR2_Ri(n)_Pbs	0.344	1.000	<0.001	0.320	0.043	<0.01
YR2_Ss_beads - YR2_Ri(n)_Pbs	<0.001	<0.001	<0.001	1.000	<0.001	0.996
YR2_Ss_Ecoli - YR2_Ri(n)_Pbs	<0.001	<0.001	<0.001	0.977	<0.001	<0.001
YR2_Ss_Pbs - YR2_Ri(n)_Pbs	<0.001	<0.001	0.886	<0.001	0.028	<0.001
YR2_Ss_Yeast - YR2_Ri(n)_Pbs	<0.001	<0.001	<0.001	1.000	<0.001	0.938
YR2_Ss_beads - YR2_Ri(n)_Yeast	0.128	<0.001	0.701	0.134	0.970	<0.001
YR2_Ss_Ecoli - YR2_Ri(n)_Yeast	0.014	<0.001	0.457	0.012	0.415	0.160
YR2_Ss_Pbs - YR2_Ri(n)_Yeast	0.084	<0.001	<0.001	<0.001	1.000	<0.001
YR2_Ss_Yeast - YR2_Ri(n)_Yeast	<0.001	<0.001	1.000	0.767	0.938	<0.001
YR2_Ss_Ecoli - YR2_Ss_beads	0.999	1.000	1.000	0.999	0.999	<0.001
YR2_Ss_Pbs - YR2_Ss_beads	1.000	0.999	<0.001	<0.001	0.986	0.122
YR2_Ss_Yeast - YR2_Ss_beads	<0.01	0.421	0.972	0.997	1.000	1.000
YR2_Ss_Pbs - YR2_Ss_Ecoli	1.000	0.999	<0.001	<0.001	0.506	<0.001
YR2_Ss_Yeast - YR2_Ss_Ecoli	0.024	0.498	0.860	0.691	1.000	<0.001
YR2_Ss_Yeast - YR2_Ss_Pbs	<0.01	0.037	<0.001	<0.001	0.967	0.325

For Figure S3. Results of multi-comparisons analyzing among different aphid genotypes; the ANOVA of whole experiment were $F_{2,6} = 9.683$; $P = 0.013$.

Multi-comparisons	PO protein
	<i>P</i> -value
LL01- YR2-Amp	0.996
LL01- T3-8V1-Amp	0.022
YR2-Amp- T3-8V1-Amp	0.020

For Figure S9. Results of multi-comparisons analyzing between un-treated and Pbs injection conditions among different aphid lines (YR2 genotype).

Multi-comparisons	Offspring number/day	Survival
	<i>P</i> -value	
YR2-Amp-untreated- YR2-Amp-Pbs	0.515	0.061
YR2-Ss-untreated- YR2-Ss-Pbs	0.750	0.371
YR2-Hd-untreated- YR2-Hd-Pbs	0.116	0.391

Attempt to knock-down PPO1 and PPO2 by RNAi

Introduction

The post-transcriptional RNA interference (RNAi) silencing technique is a powerful tool to transiently inactivate the expression of targeted genes in animals. It was first reported in the nematode *Caenorhabditis elegans* (Fire et al. 1998), allowing a very specific silencing of the target gene. In 2006, siRNA was used in pea aphid, inducing the silence of a target gene (the C002 transcript) in the salivary glands of the adult aphid (Mutti et al. 2006). Then, a long dsRNA was used to trigger the silencing (with a comparable 40% decrease) of two marker genes (Ap-crt and Ap-cath-L) in the gut of third-instar pea aphids (Jaubert-Possamai et al. 2007). Recently, a study has shown that phenoloxidase can also be partially inhibited by RNAi in pea aphids (Xu et al. 2019). In order to complete the work described in Publication 3, we decide to use RNAi to knockdown each PPO gene of the pea aphid. This approach could be helpful to answer several questions such as, i) do the two PPOs circulate as we suggest, ii) are they equivalent in terms of activity in the hemolymph, iii) are they both expressed in the hemocytes.

Materials and methods

dsRNA synthesis

The PCR primers were designed using the NCBI primers designing tool Primer-Blast (Table 9) and the T7 promoter sequence was added to the primers. They were used to prepare double-stranded RNA fragments of PO2 and PO2-X1 (Tomoyasu et al. 2005). Briefly, we used the synthesized cDNA of pea aphids as a template, then the corresponding PCR products were sequenced to ascertain their quality and purified via a Gel Extraction kit (Qiagen, www.qiagen.com). The purified product was used as the template for the synthesis of dsRNA following the procedure of the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). The quantity and quality of the dsRNA was examined by nanodrop (Agilent) and agarose gel electrophoresis. Double-stranded luciferase RNA (dsLuc) was used as the control. All dsRNAs were diluted to nearly 350 ng/μl and stored at -20 °C.

Injection

Injection of dsRNA: 5-day-old nymphs (L3) (Strain: symbiont free YR2-Amp,) were collected, then 46 nl of dsRNA were injected into the hemocoel (Simonet et al. 2016) using a Nanoject III micro-injector (Drummond Scientific, Broomall, PA, USA) with glass capillaries prepared on a Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). The aphids were

transferred and maintained on *Vicia faba* plant (14-day-old) at 20 °C and 16:8 h light/dark cycle until collection at each time point after injection (i.e. the following 1st day, 3rd day and 6th day).

Estimation of transcript levels

The efficiency of RNAi was determined by measuring the relative expression levels of PO2 and PO2-X1. Total RNA was isolated from individual pea aphid using RNeasy plus micro kit following the manufacturer's instruction (Qiagen, Germany) and quantified on a nanodrop (Agilent). cDNA was generated from 500 ng RNA using iScript cDNA synthesis kit (Hercules, California, USA), according to the manufacturer procedures. Specific mRNA primers (i.e. for PO2, PO2-X1 and EF1) and assay conditions for RT-PCR were as described in Luo et al. (In prep; Article 3). Three biological replications were done.

Table 9. Primers used in the study of dsRNA synthesis.

Primer names	Primer sequences	Product size (bp)
dsPO2-1-F	*AACAGGTTCAAGAACCCCGA	488
dsPO2-1-R	*GGAAGTGTGACCAGCCGTA	
dsPO2-X1-F	*GGGAACCAGTAATAACAACAAG	403
dsPO2-X1-R	*GGTCGAATAGTCAATGTAGGT	

*These are preceded by the T7 adaptor TAATACGACTCACTATAGGGAGA for dsRNA synthesis

Results

Under our conditions, the expression of the pea aphid PO2 or PO2-X1 genes was unaffected after injection of double-strand RNA. The level of expression of the two PO genes was similar between injected aphids and controls (dsLuc injected) at 1st, 3rd and 6th days after injection of double-stranded RNA (dsRNA). Due to the similarities of the sequences, we tested the effect of dsRNA on the expression of both POs. The results showed that dsPO2 did not decrease the expression of PO2 (Fig. 23 A; $F_{1,4}= 0.036$, $P= 0.814$; $F_{1,4}= 7.168$, $P= 0.055$; $F_{1,4}= 0$, $P= 0.998$ for the 1st, 3rd and 6th days, respectively) nor that of PO2-X1 (Fig. 23 B; $F_{1,4}= 0.218$, $P= 0.665$; $F_{1,4}= 0.006$, $P= 0.943$; $F_{1,4}= 0.291$, $P= 0.618$ for the 1st, 3rd and 6th days, respectively). Similarly, the injection of dsPO2-X1 did not reduce the expression of PO2-X1 (Fig. 24 A; $F_{1,4}= 0.007$, $P= 0.939$; $F_{1,4}= 0.04$, $P= 0.852$; $F_{1,4}= 0.115$, $P= 0.752$ for the 1st, 3rd and 6th days, respectively) and PO2 (Fig. 24 B; $F_{1,4}= 0.056$, $P= 0.825$; $F_{1,4}= 0.001$, $P= 0.977$; $F_{1,4}= 0.347$, $P= 0.587$ for the 1st, 3rd and 6th days, respectively).

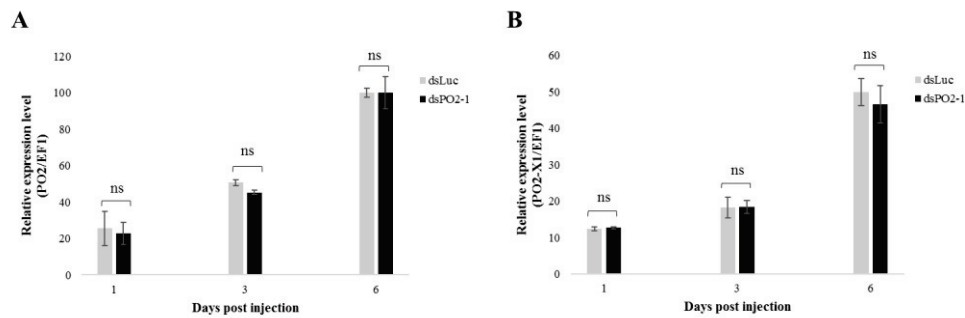


Fig. 23. Efficiency of RNA interference-mediated knockdown of pea aphid prophenoloxidase 2 (PO2). The relative expression levels for (A) PO2 and (B) PO2-X1 were analyzed by quantitative RT-PCR post-injection. Graphs display the mean value \pm SEM in messenger RNA levels relative to the double-stranded luciferase gene of a firefly (dsLuc)-injected group. “ns” means no significant difference; n=3.

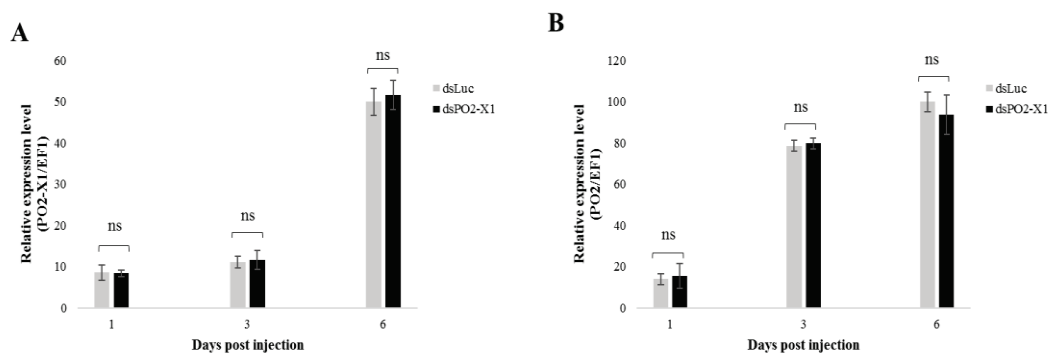


Fig. 24. Efficiency of RNA interference-mediated knockdown of pea aphid prophenoloxidase 2-X1 (PO2-X1). The relative expression levels (A: PO2-X1; B: PO2) were analyzed by quantitative RT-PCR post-injection of double-stranded PO2 (dsPO2-1) in the aphids. Graphs display the mean value \pm SEM in messenger RNA levels relative to the double-stranded luciferase gene of a firefly (dsLuc)-injected group. “ns” means no significant difference; n=3.

Discussion

RNA interference (RNAi), a post-transcriptional gene silencing mechanism, is an important research tool for determining gene functions. Here, our attempt to silence the pea aphid POs by RNAi was unsuccessful. Phenoloxidase (PO) is a key component of the insect immune system and previous experiments with different insect species showed that phenoloxidase could be silenced (Binggeli et al. 2014; Zhang et al. 2019). In pea aphids, the first study of gene knockdown by RNAi resulted in a 40% decrease in targeted gene expression (Jaubert-Possamai et al. 2007). Time-course analysis of the silencing showed that inhibition reached its maximum 5 days after injection and ended after 7 days. Since then, several other studies have been done with different outcomes, some having worked (Niu et al. 2019) and others, like ours, were unfruitful (Christiaens et al. 2014).

We were particularly disappointed because a recent publication reported a silencing of POs in pea aphid by micro-injection of a very low dose of about 0.4 ng of dsRNA per aphid (Xu et al.

2018). In their study, PO2-X1 or the two POs together (i.e. PO2+PO2-X1) were silenced by more than 50%. PPO2 expression significantly decreased at days 3 and 5 post-injection, and, with the dsRNA which targets a common region of PPO1 and PPO2, the expression of PPO1 and PPO2 was the lowest on the third day. However, in both cases, the activity of PO in the hemolymph did not seem drastically affected, suggesting a sufficient amount of protein to maintain it. They also reported that inactivation of the PPO gene resulted in an increase in the number of live bacterial cells and fungal spores in the body of aphids and higher mortality after infection.

Possible explanations for failure may be multiple and could include 1) dsRNA injection dose: previous success of RNAi in pea aphid identified a minimum dose of injected dsRNA of at least 28 ng per individual (Jaubert-Possamai et al. 2007), a dose of 50 ng being more frequently used (Mutti et al. 2006; Sapountzis et al. 2014). Niu et al. (2019) required a dose of 60 ng dsRNA per pea aphid to significantly silence the gene *Aphunchback*. All this suggests that successful RNAi in pea aphid may be gene dependent (due to the expression timing) and requires enough dsRNA to be injected and persist in the hemolymph. In our study, we could inject only 16 ng dsRNA per aphid, because of the difficulty to obtain a high concentration of dsRNA of PO2 or PO2-X1. However, the fact that Xu et al. (2018) obtained a knockdown with a very low dose of dsRNA may suggest a problem of too much injected quantity rather than an insufficient amount.

Another difference between our study and that of Xu et al. (2018) is the dsRNA sequences and the length of these sequences (our sequences were much longer), since the dsRNA sequence and the dsRNA fragment size appear to be important factors for cell uptake and the effectiveness of the silencing (Huvenne et al. 2010; Bolognesi et al. 2012). At last, the aphid genotype used is different and the presence of symbionts in their aphid line was not tested. This may also affect the efficiency of injected-dsRNA in aphids. The sequence is also important for the degradation of dsRNA after injection; Christiaens et al. (2014) suggested that the injected dsRNA would degrade in a few hours and become almost undetectable in 24 hours in pea aphids. We did not test such a hypothesis for our PO experiment. On this basis, the next steps in our experiment should be a) designing a shorter dsRNA and testing higher and lower dose; b) examine the efficiency of the silencing at different times after treatment; c) try another route of dsRNA delivery, such as feeding or topical dsRNA delivery (i.e. spraying) method. A recent study suggested that topical dsRNA delivery had superior gene silencing efficiency compared to the microinjection method (Niu et al. 2019).

Short-term effects of secondary symbionts on pea aphid immunity

In Publication 3, we have used long established lines, either cleaned of facultative symbiont or resulting from infection by microinjection of this symbiont-free background with different symbionts. We took advantage of these different lines to initiate a further study on the short-term impact of infection with different facultative symbionts on the pea aphid's immunity (mainly PO). Indeed, most studies published to date have used mainly *E. coli* or other pathogenic bacterial strains to analyze their effect on pea aphid immunity. However, it is suggested that facultative symbionts can be transferred horizontally between aphids in the wild (Oliver et al. 2010; Li et al. 2018), making it interesting to study a more biological situation. This study also provides a tool to estimate the time required for adaptation between the aphid and symbionts. In this context, we followed these newly created lines for several generations to obtain an indication of this adaptation time.

This work, although still in progress, is presented here in a draft publication format.

Short-term effect of symbionts infection on pea aphid immunity and life history traits.

Chen Luo et al.,

Abstract

Aphid individuals can harbor one or more of nine facultative symbionts, the most frequent being *Hamiltonella defensa* (*Hd*), *Regiella insecticola* and *Serratia symbiotica* (*Ss*). We have recently shown that bacterial symbionts can manipulate their host immune system. However, the pea aphids used in this study were experimental lines established more than 10 years ago and therefore had time to undergo a co-adaptation. Herein, to estimate how long this adaptation took, we artificially created three new lines of pea aphid with the same genetic background (YR2-amp), one injected with PBS and used as a control (YR2-PBS), the others infected with the symbiont (*Ss*) or (*Hd*) from YR2 long infected lines. The results showed that the presence of *Hd* and *Ss* strongly increases the amount of PO during the first generation, then a decrease occurs, and the level becomes equivalent to the control after the 2nd generation (G2). For *Hd* but not for *Ss*, the amount of PO decreases further and at the 5th generation, it becomes very low and even lower after. In addition, we found that at the 6th generation, PO gene expression and PO activity, as well the number of offspring and the survival rate were reduced by the presence of *H. defensa*, but not *S. symbiotica*, a situation similar to that observed in long-term established lines. Therefore, we conclude that the effect of the infection by endosymbiotic bacteria on the host immune system is a biphasic process characterized by short-term induction followed by a return to equilibrium after a few generations at a level that is dictated by the symbiont.

Key words: secondary symbiont; aphids; immunity; phenoloxidase; fitness; fast co-evolution.

Introduction

Insects depend almost entirely on their innate immune system to cope with challenging biotic conditions, because they do not have an adaptive immune system (McFall-Ngai 2007; Hillyer 2016). Annotation of the pea aphid genome has shown that many components essential for immune function in other insects are missing, such as the antimicrobial peptides (AMPs), cell pattern recognition receptors (PRRs), peptidoglycan recognition receptors (PGRPs) and many of the components of the immunodeficiency (IMD) pathway (Gerardo et al. 2010). In addition to the obligate symbiont *Buchnera aphidicola*, which performs a nutritional function (Douglas 1998; Moran et al. 2008), pea aphids can also harbor a number of facultative symbionts in secondary bacteriocytes, hemolymph or immune cells (Oliver et al., 2010; Schmitz et al. 2012). The hosts initially acquire these secondary symbionts via horizontal transmission; once established, they are transmitted vertically from parents to offspring (Russell and Moran 2005; Oliver et al. 2010; Henry et al. 2013). Since aphids are symbiotic organisms, some authors have hypothesized that mutualism with facultative bacterial symbionts could alter the limited immune system of aphids by conferring an extended defensive phenotype (Oliver et al. 2010; Sudakaran et al. 2017; Welchman et al. 2009).

Our previous studies have shown that facultative symbionts can manipulate the pea aphid cellular (e.g. the number of adherent hemocytes, the proportion of two adherent phagocytic cell types, granulocytes and plasmatocytes) (Schmitz et al. 2012) and humoral immune system (e.g. phenoloxidase expression and activity) in different aphid genetic backgrounds (Luo et al. in prep). Meanwhile, in agreement with other studies (Laughton et al. 2016), our results showed that the genotype of the aphid had little effect on the result of the immune response compared to the bacterial effect (Schmitz et al. 2012; Luo et al. in prep). However, in these previous studies, we used experimental aphid clones created more than 10 years ago (equivalent of more than 250 generations in the laboratory) by symbionts transfer (Koga et al., 2007; Simon et al., 2011). Here, we carried out an experiment using the same pea aphid genotype (YR2-amp) transfected with two symbionts (*Hd* and *Ss*) by micro-injection. These two symbionts were chosen because the long-term presence of *Hd* in the background YR2 had a strong effect on aphid immunity, unlike *Ss*. The results clearly showed that, although infection by both symbionts affects immunity with a strong induction of PO at the first generation, the situation has returned to the control level after the 2nd generation. After the 4th generation, the situation has returned as that of long-term established lines: the presence of *Hd* presence, but not that of *Ss*, strongly decreases the PO compared to the control line and affects the reproduction and survival of the host.

Materials and methods

Aphids lines

The lines used in the study are long-term established experimental lines, namely: The YR2-Amp clonal line, devoid of facultative symbionts, created by antibiotic treatment of the field-originating line harboring *R. insecticola*, and YR2Ss and YR2-Hd obtained by injecting this strain with *S. symbiotica* and *H. defensa* from donor clones (Koga et al., 2007; Simon et al. 2011).

Creation of new lines

The lines YR2-Amp-Ss and YR2-Amp-Hd were created by micro-injection into the body of a 3rd-instar (5-dayold) YR2-Amp aphid of PBS diluted hemolymph (1:1) obtained from YR2-Ss or YR2-Hd aphids with a Nanoject II apparatus (Drummond) set up for a volume injection of 23nl. As a control, the same volume of PBS was injected in YR2-Amp aphids (YR2-Amp-PBS). Each injected aphid was transferred to an individual 9-cm Petri dish lined with a moistened filter paper and containing a freshly cut fava bean leaf with the stem in wet cotton wool wrapped with aluminum foil. Once the aphid started to reproduce, 10 of its offspring were tested by PCR for the presence of the symbiont and when 100% were infected, three of their sisters were collected to create the three different lines for each condition (G1 generation). We routinely screened offspring of different generations by PCR as reported previously (Desneux et al. 2018; Monticelli et al. 2019) to ensure the persistence of corresponding symbionts. All aphid lines were maintained on *Vicia faba* plants at 20 °C and 16:8 h light/dark cycle.

Experimental procedures

Sample collection: For the following experiments, we collected 13-day-old synchronized adults from the 1st generation (G1) to the 10th generation (G10) (synchronization as previously described (Schmitz et al., 2012)).

PO protein quantity assay: Pools of five 13-day-old aphids were collected from each generation from the three biological replicates. The method of quantification of PO has been previously described (Luo et al., in prep). Briefly, the aphids were cleaned with a 70% ethanol bath, rinsed with distilled water, and immersed in a drop of 10 µl of Ringer solution (KCl 182 mM; NaCl 46 mM; CaCl₂ 3 mM; Tris-HCl 10 mM) supplemented with a protease inhibitor cocktail (Sigmafast; Sigma) per aphid on a Petri dish over ice. Then, the ventral cuticle was carefully ruptured under a stereomicroscope, and the diluted hemolymph was collected and immediately centrifuged at 15,000g for 10 min. The supernatant was carefully collected and mixed with Laemmli buffer containing β-mercaptoethanol and then boiled. To compare the

amount of PO protein in the hemolymph, the proteins were first separated on 12.5 % SDS-PAGE and the gel was silver stained (Morrissey, 1981) to evaluate the total amount of protein and equalize the samples. The adjusted protein quantities were loaded on two 12.5 % SDS-PAGE, one was silver stained and photograph using a digital camera (EOS5D, MKII; Canon) and the other was transferred on a 0.2 μ m nitrocellulose membrane (Amersham). The membrane was blocked with TBS-Tween 2% low fat milk, then incubated overnight at 4 °C with an “in-house” rabbit polyclonal antibody against aphid PO (Luo et al., in prep). To probe the Western blot, the serum was used at a final dilution 1/2500. After three washes in TBS, the membrane was incubated for 2 hours with Goat anti-rabbit-HRP (1/10000; Sigma) at room temperature. The signal was revealed using a Luminate Crescendo Western HRP substrate (Merck Millipore) and digital pictures obtained with an imaging device (Chemi Genius2; SynGene). The total intensity of each lane of the silver stained gel and of each reactive band on the Western blot were quantified using Image J software. Finally, the intensity of the reactive band was divided by the intensity of the corresponding silver stained lane to correct for the variation of the amount of charge between the lanes.

PO2 and PO2-X1 gene expression: Five 13-day-old aphids were collected from the 6th generation (G6) and pooled. Extraction was done separately for the three biological replicates previously described (Luo et al. in prep). Briefly, total RNA was isolated from pooled aphids using the RNeasy-plus micro kit following the manufacturer’s instruction (Qiagen, Germany) and quantified on a nanodrop (Agilent). The cDNA was generated from 500 ng RNA using the iScript cDNA synthesis kit (Hercules, California, USA), according to the manufacturer procedures. Specific mRNA primers used for RT-PCR were previously described (Luo et al., in prep) (Table 1). PCR was performed in 25 μ l containing 5 μ l 5x green reaction buffer (Madison, Wi USA), 1 μ l forward primer, 1 μ l reverse primer, 2.5 μ l 5mM MgCl₂, 0.5 μ l 20mM dNTP, 0.125 μ l GoTaq DNA polymerase (Madison, Wi USA), 1.5 μ l cDNA (100ng/ μ l) and nuclease-free water. The PCR cycle parameters were as followed: 95 °C for 4 min and 30 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, then 72°C for 10 min. The resulting PCR products were separated in a 1.5 % agarose gel with ethidium bromide and visualized under UV. Image J software was used to obtain the intensity of the PCR bands. The intensity of the PO bands was divided by the intensity of the EF1 band of the same sample to estimate the level of gene expression. The controls were done by replacing the RT products by the same volume of H₂O in the final mix.

PO activity assay: Five 13-day-old aphids were collected from the 6th generation (G6), and more than 7 replicates were made for each line. Briefly, PO activity was tested by its catalytic

conversion of L-dopa (3,4-dihydroxy-L-phenylalanine; Sigma) to dopachrome (Saul et al., 1987). Measures were directly performed on the pooled hemolymph of five aphids. Hemolymph was collected directly into a 70 μ L drop of distilled water, then diluted hemolymph was immediately transferred into a 0.5 mL centrifuge tube and centrifuged (10,000g, 1 min, 4 °C). The supernatant was collected and centrifuged again under the same condition. Finally, 50 μ L was collected, immediately frozen in liquid nitrogen and kept at -80°C until the PO activity test. Before use, the samples were defrosted on ice and vortexed. Then, 15 μ L of sample were mixed with 150 μ L of distilled water (as a control) or 150 μ L of L-dopa solution (1mg/mL in distilled water; two technical replicates per sample) in a 96-well plate. The reaction was monitored at 30 °C in a spectrophotometer (Versamax, Molecular Devices) by reading at 490 nm every 30 seconds for 1 hour. Activity curves were analyzed using Softmax Pro v 4.0 software. The enzymatic activity (V_{max} in OD/min) was considered as the maximum linear slope during the conversion of the substrate.

Life-history traits assay: 13-day-old aphids were collected from the 6th generation (G6), and 5 replicates were made per line. Pools of five YR2-Amp-PBS, YR2-Amp-Ss and YR2-Amp-Hd aphid were transferred to individual 9-cm Petri dish lined with a moistened filter paper and containing a freshly cut fava bean leaf, as described. 24 hours later, we began recording the number of offspring (counted and removed) and the number of dead aphids each day until the death of all individuals. Leaves and filter papers were replaced every 3 days. The experimental conditions were 20 °C and 16:8 h light/dark cycle.

Analyzes. All statistical analyzes were performed in R version 3.4.3. All proteins, gene expression, enzymatic activity and number of offspring were analyzed by performing ANOVA, after validation of the normal distribution of the dependent variable. Aphid survival was analyzed using the proportional hazards model (Cox, 1972), and a survival package was used (Therneau, 2015). The ‘multcomp’ package was used to perform multiple comparisons using Tukey’s contrasts. All data and complementary explanations are in Supplementary File 1.

Results

The amount of PO protein in the hemolymph was compared between YR2-Amp-Pbs (control), YR2-Amp-Ss and YR2-Amp-Hd (Fig. 1) by combining silver stained gels and quantification by Western blot, as described in the Materials and Methods. G1 offspring of the aphids inoculated with *H. defensa* and *S. symbiotica* had a significantly higher amount of PO protein than PBS-injected aphids (Table S1). In the next generation (G2), a marked decrease in PO occurred in *Hd*- and *Ss*- infected aphids since the amount reached the same level as that of the

control. In contrast to *Ss*, in which the amount of PO seemed to stabilize after G2, a decrease in PO occurred at G5 in *Hd*-infected aphids and appeared to continue until G7-G8, with a protein level that has become almost undetectable.

Since *Ss* was lost at generation G7, we used the G6 generation which seemed to be the first generation where a steady state was reached to compare the expression of the two PO genes (Fig. 2a and 2b) and the PO activity (Fig. 2c). The results agree with those on the amount of protein: in the presence of *H. defensa*, but not *S. symbiotica*, the expression of the PO2 and PO2-X1 genes was reduced, as well as the PO activity compared to the control (Table S2). In addition, we also found that the presence of *H. defensa* (Fig. 3a) but not that of *S. symbiotica* (Fig. 3b) could significantly reduce reproduction and survival rates of aphid hosts (Table S3), a situation previously observed in the presence of *Hd* in the long-term established lines (Simon et al., 2011; see publication 3 also).

Discussion

The fitness of an organism depends primarily on its immune system to provide protection against parasites and pathogens (Schulenburg et al., 2009; Sadd and Schmidt-Hempel, 2009). Insects have an innate immune system articulated around two components: a humoral component comprising antimicrobial peptides, lytic enzymes and enzymatic cascades like that of phenoloxidase and a cellular component consisting of different hemocytes with their specialization (e.g. phagocytosis, encapsulation). For many invertebrates, symbionts provide host protection against pathogens and parasites. The basis of this extended immunity phenotype is beginning to be deciphered for parasitoids but remains unknown in many aspects. For example, the way the insect immune system handles hosted symbionts is still not well understood. We previously showed that symbionts could selectively impact the immune system of pea aphids, including on the expression of PO. (Luo et al., in prep). Here we tested the effect on immunity of successive generations after the transfer of symbionts. This can be an important issue as more and more work are done on this model, sometimes with a short delay between line creation and experimentation. Interestingly, only the first generation (G1) from aphids infested with *H. defensa* or *S. symbiotica* showed a large increase in the amount of PO protein suggesting an immune response of the host. In our previous study, the injection of *E. coli* and yeast induced a PO response in the YR2-amp line, but that of latex beads did not (Luo et al., in prep). Thus, it will be possible to determine with further studies whether the response observed at G1 is based on general mechanisms which are not specific of symbiont species. The way by which this response may occur is uncertain since genome analysis suggests that pea aphid has

no or very few Pathogens Related Receptors, such as PGRPs and GNBPs, capable of recognizing bacterial components (Gerardo et al. 2010). One hypothesis is that other bacterial components could trigger the PO response. In pea aphid, PO has been localized in some hemocytes (Schmitz et al 2012; Laughton et al. 2011) while bacterial infection is known to increase the number of circulating hemocytes (Laughton et al. 2011). Interestingly, aphid hemocytes are capable of phagocytosing circulating symbionts (Schmitz et al. 2012). Whether these hemocytes are the producers of this PO remains to be determined. The studies are difficult because, to date, no hematopoietic organ or PO-producing tissue has been described in this species.

In the following generation G2, G3 and G4, the PO protein amount was the same between the treatments (YR2-Amp-Hd or YR2-Amp-Ss) and the control (YR2-Amp-Pbs) suggesting a quick acceptance/adaptation of the host physiology to the secondary symbionts. However, from G4 to G7, the lines with *Hd* showed a further decrease in PO, as if the symbiont took control of the host by controlling its own immunity. The level of PO then reached by the *Ss* and *Hd* aphid lines was similar to that observed in the long-term established equivalent lines (Luo et al, in prep).

This was confirmed by the results on the expression and activity of the PO2 or PO2-X1 gene. In addition, we found that the fecundity and survival of aphid lines newly infested with *H. defensa* would also be reduced as in the long-term established *Hd* lines. Interestingly, there was a potential relationship between PO expression and host fitness traits, as in *Drosophila* (Binggeli et al., 2014).

Our results therefore strongly suggest that the effect of the symbiont on the host immune response occurs rapidly (within a few generations) and the final level of the effect depends primarily on the symbiont. The data also confirmed that the long-term impact of *H. defensa* on PO levels is certainly mediated by regulation of transcription and/or translation of PO. It will be interesting in the future to introduce these impacting symbionts in different species of aphids to analyze their effect on the immune components. A better characterization of the *H. defensa* strain used (particularly the presence and type of APSE) will also help to decipher the mechanism involved in the regulation of the PO gene.

Another aspect is the time required for the symbiont to colonize the host. In aphids, all types of symbionts are found at three locations within the host: secondary bacteriocytes, sheath cells, and hemocoel (Moran et al., 2005). It may take one or two generations of aphids before the symbionts enter the bacteriocytes and sheath cells. During this time, they are more present in the hemolymph and thus likely to induce an immune response. Conversely, it could be the time

needed for the aphid to develop specific bacteriocytes and sheath cells. Recently, aphid genotypes from different biotypes have been shown to vary in acceptability and maintenance of symbionts after horizontal transfer (Parker et al., 2017). Biotypes that frequently host symbionts are better able to associate with new symbionts than biotypes that host symbionts less frequently. This may suggest that once aphids have been in contact with the symbiont, they may have coevolved and are less prone to develop a strong immune response. It will be interesting to replicate this experiment with a line that has never hosted a facultative symbiont such as LL01 to see if it will take more generations to reach the level of immunity at equilibrium.

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Table 1. Primers used in the study.

Target	Primer name	Primer sequence 5' to 3'	Amplicon size (bp)
Phenoloxidase 2 (PO2)	PO2-paF	GGCAGTGGTTCAAACAGGTT	173
	PO2-paR	TTACGATATCCACCGGTCCG	
Phenoloxidase 2-X1 (PO2-X1)	PO2-X1-paF	CTTGTTTCGATCGTCCTACCG	194
	PO2-X1-paR	CCCAGTGGAATCGACAAGTC	
Elongation factor 1 (EF1)	EF1-paF	GGCCGACGGTAAATGTTTGA	232
	EF1-paR	CAAAGCTTCGTGGTGCATCT	

FIGURES

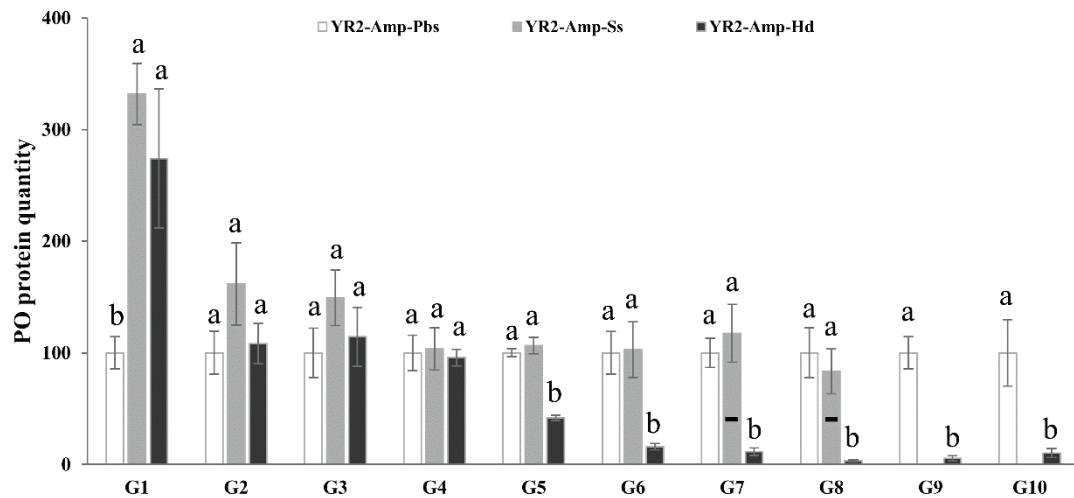


Figure 1. PO protein quantity from the first generation (G1) to the tenth generation (G10). Parental lines were created by injection of PBS (control), *Hd* or *Ss* into the YR2-amp background. *Hd* was obtained from YR2-Hd and *Ss* from YR2-Ss, both long-term established lines. The PO quantity was measured and expressed as described in the materials and methods. Results expressed as mean \pm SEM and different letters (a, b) indicate statistically significant differences ($p < 0.05$; multi-comparison test from “multcomp” package) ($n = 3$). The (-) sign in the columns indicates the loss of the symbiont *S. symbiotica* in the line at G7.

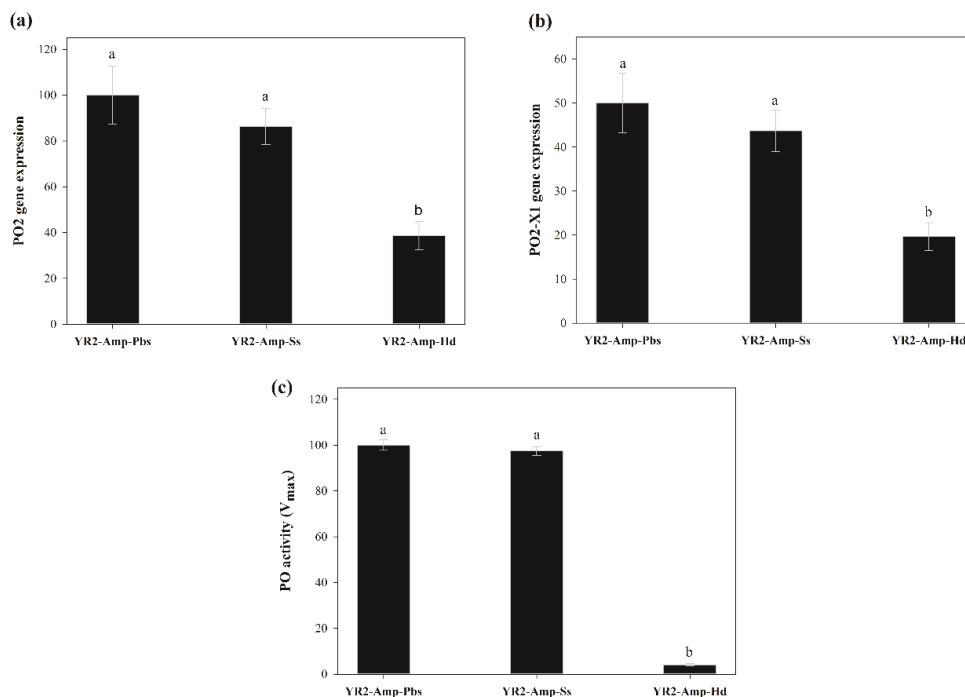


Figure 2. PO genes expression and PO activity of the lines at the 6th Generation.

The level of expression of PO2 (a) and PO2-X1(b) was measured by PCR in the aphid lines YR2-Amp-Pbs (control), YR2-Amp-Ss and YR2-Amp-Hd at sixth generation (G6). Results expressed as a ratio between the expression of the PO gene and the EF1 reporter gene. (c) Hemolymph PO activity of the three aphid lines measured spectrophotometrically by conversion of L-DOPA. All results expressed as mean \pm SEM and different letters (a, b) indicate statistically significant differences ($p < 0.05$; multi-comparison test from “multcomp” package). $n = 3$ for gene expression and $n = 7$ for the activity.

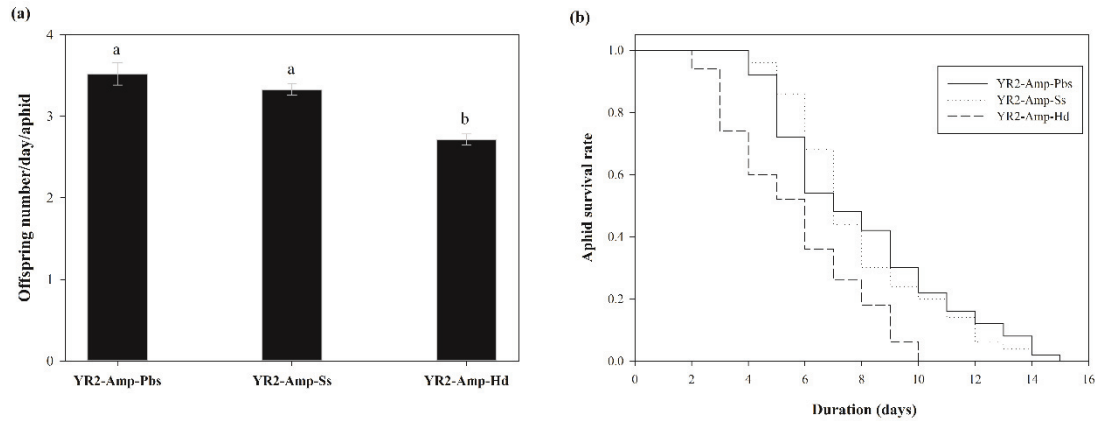


Figure 3. Comparisons of fitness traits among created aphid lines at G6. (a) reproductive rate; (b) survival rate. Results expressed as mean \pm SEM and different letters (a, b) indicate significant differences ($p < 0.05$; multi-comparison test from “multcomp” package), $n = 5$.

Supplementary materials

Supplementary File 1

Table S1: Statistics for Figure 1. Results of multi-comparisons analyzing different aphid lines from G1 to G10 on the amount of PO protein. The ANOVA data of whole experiment were $F_{2,6} = 9.056$, $P = 0.015$ (G1); $F_{2,6} = 1.619$, $P = 0.274$ (G2); $F_{2,6} = 1.072$, $P = 0.399$ (G3); $F_{2,6} = 0.068$, $P = 0.935$ (G4); $F_{2,6} = 50.756$, $P < 0.001$ (G5); $F_{2,6} = 7.365$, $P = 0.024$ (G6); $F_{2,6} = 11.336$, $P = 0.009$ (G7); $F_{2,6} = 8.847$, $P = 0.016$ (G8); $F_{1,4} = 41.823$, $P = 0.003$ (G9); $F_{1,4} = 8.918$, $P = 0.004$ (G9).

Multi-comparisons	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
	<i>P</i> -value									
YR2-Amp-Pbs - YR2-Amp-Ss	0.016	0.295	0.390	0.986	0.651	0.994	0.758	0.784		
YR2-Amp-Pbs- YR2-Amp-Hd	0.049	0.974	0.916	0.977	< 0.001	0.039	0.023	0.018	0.003	0.004
YR2-Amp-Ss- YR2-Amp-Hd	0.594	0.382	0.593	0.929	< 0.001	0.035	0.010	0.040		

Table S2: Statistics for Figure 2. Results of multi-comparisons analyzing different aphid lines at G6 on PO gene expression and PO activity. The ANOVA data of whole experiment were $F_{2,6} = 11.756$; $P = 0.008$ (PO2 gene expression); $F_{2,6} = 9.889$; $P = 0.013$ (PO2-X1 gene expression); $F_{2,20} = 875.440$; $P < 0.001$ (PO enzyme activity).

Multi-comparisons	PO2 gene	PO2-X1 gene	PO activity
	<i>P</i> -value		
YR2-Amp-Pbs - YR2-Amp-Ss	0.582	0.673	0.551
YR2-Amp-Pbs- YR2-Amp-Hd	0.009	0.013	< 0.001
YR2-Amp-Ss- YR2-Amp-Hd	0.027	0.036	< 0.001

Table S3: Statistics for Figure 3. Results of multi-comparisons analyzing different aphid lines on aphids’ life-history traits. The ANOVA data of whole experiment were $F_{2,12} = 18.966$; $P < 0.001$ (reproductive rate); $X^2 = 7.332$; $P = 0.026$ (survival rate).

Multi-comparisons	Offspring number/day	Survival
	<i>P</i> -value	
YR2-Amp-Pbs - YR2-Amp-Ss	0.362	0.989
YR2-Amp-Pbs- YR2-Amp-Hd	< 0.001	0.033
YR2-Amp-Ss- YR2-Amp-Hd	0.002	0.044

Change in PO during the aphid development varied upon the hosted symbiont

During parthenogenetic reproduction, aphid travel through four nymphal stages (instars) from birth to adulthood, and they undergo an incomplete metamorphosis between each stage. During these stages of development, the number of *Buchnera* and bacteriocytes change (Fig. 25), both increasing strongly and concomitantly to reach a maximum in young adults (aged 9-13 days), before a decrease due to the death of the bacteriocytes (Simonet et al. 2016 2018).

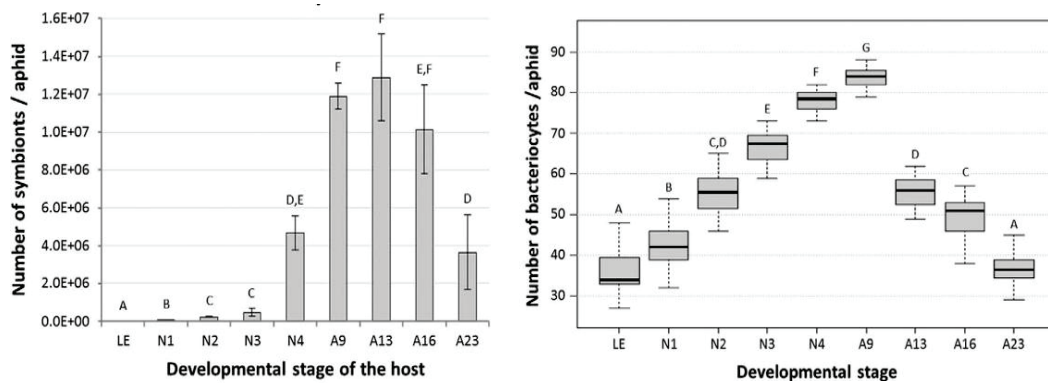


Fig. 25. Dynamics of *B. aphidicola* population and bacteriocytes number during aphid development. Left: Variation in the number of symbiont cells, quantified by flow cytometry analysis from whole aphids. Right: variation in the number of bacteriocytes per aphid, in relation to host developmental stage. LE, late embryos; N1 to N4, nymphal stages from 1 to 4; A9-A23, adult time points from day 9 to day 23. (from Simonet et al. 2016).

There is much less information about the facultative symbiont variation during development. In their study, Doremus and Oliver (2017) indirectly estimated that the *H. defensa* titer increased sharply during nymphal development (between the 2nd and 3rd instar) and then decreased slightly a little before reaching the adult stage (A8 (192h)) (Fig. 26).

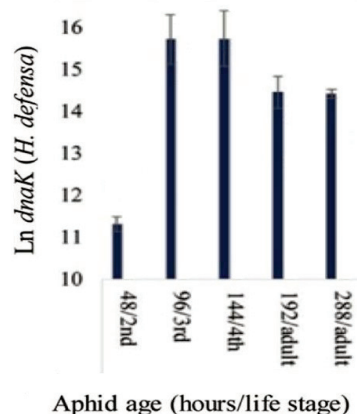


Fig. 26. Natural log values of mean estimated *H. defensa* *dnaK* gene copies within aphids across five developmental time points.

In addition, a recent study on *Sitobion miscanthi* suggested that the presence of *Hd* could stimulate the proliferation of *B. aphidicola* and increase the fitness of the host (Qian et al. 2018). Although the absolute number of *Buchnera* and facultative symbionts may vary with the genotype of pea aphid and external conditions (Vogel and Moran 2011; Enders and Miller 2016; Chong and Moran 2016), significant changes occurred during aphid development. In view of our previous data, it is interesting to analyze the relationship between these variations and host immunity. To our knowledge, there is no information on innate immune components (hemocytes and PO) during aphid development published to date.

In the laboratory, we obtained preliminary results showing that the number of adherent cells varied during the developmental stages and was not related to the size/weight of the aphid (Fig. 27).

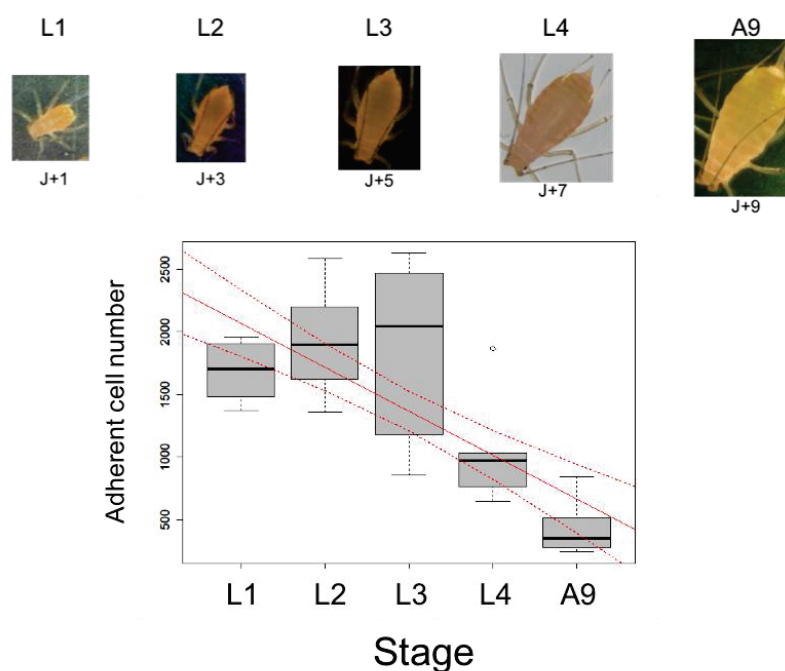


Fig. 27. Development stage and number of adherent cells in YR2-Amp pea aphid. Upper, the different stages of development are shown on the same scale with up the stage and down the number of days since birth (L= nymphal larvae; A= adult). Lower: the average number of total adherent hemocytes counted as described in Schmitz et al. (2012) obtained at the indicated stage of development. (From J. Villalba)

In this example, it appears that the number of cells is higher in the first nymphal stage and then decreases. One possible explanation is that the hemocytes renewal ending after stage 3 may be due to nutritional competition with increasing numbers of symbionts. Whatever the precise reason, it was interesting to check whether humoral components of aphid immunity, the PO, followed the same developmental trend.

I have therefore analyzed the variations in the amount of PO and in the expression of the two PO genes throughout the development of the LL01, YR2-Amp, YR2-Ss, YR2-Ri(n) and YR2-Hd lines (Fig. 28; Statistical analysis, Table 10). The results identified two developmental groups with a similar pattern of changes in the amount of PO and gene expression: In the first group, LL01, YR2-Amp and YR2-Ss, the level of PO keeps increasing from early stages to adult (Fig. 28 a, c, e). In the second group, YR2-Ri(n) and YR2-Hd, both the amount of PO and the expression of the genes increase at the early stages (L1-L3), then significantly decrease at the 4th-instar stage to reach a very low level in the young adult (Fig. 28 b, d, f).

Our results therefore explain the difference in PO observed in adult pea aphid in the presence of certain symbionts (*Hd*, *Ri*). This difference comes from nymphal development. In the third stage something happens in the lines *Hd* and *Ri*, but not in the lines *amp* and *Ss*, which behave like LL01, a line naturally free of facultative symbionts. The sharp increase in *Buchnera* number and bacteriocyte size occurs at this stage and may require a lot of nutrients, but it should occur in the different lines. Since the number of *Hd* also increases at this stage (we think that it may be the same for *Ri* although it remains to be confirmed), it is possible that the toxins released by these bacteria or the competition for specific nutrients affect PO production (via the cells/tissues that produce the PO). *Serratia symbiotica* has been shown to be less pathogenic and free of toxins present in *Hd* and *Ri* (Degnan and Moran 2008b; Degnan et al. 2009, 2010), and its presence induces only small variations in the expression of 28 genes in the host (Burke and Moran 2011). This may explain the different responses to symbionts and support our hypothesis. At last, if only a few percent of the hemocytes can produce PO (Laughton et al. 2011; Schmitz et al. 2012), a change in their number, illustrated in Fig. 27, could be related to the decrease in the enzyme. However, in this case, the YR2-amp shows a stable increase of PO all along the development while hemocytes decrease after L3, so it is unlikely that the few percent (about 4-5%) of the PO-positive hemocytes remaining at adult stage (Schmitz et al. 2012) are the main producer of PO in aphids. Recently, a study on a social aphid *Nipponaphis monzeni* showed that when enemies damage their plant-gall nest, nymphs of soldiers discharge a large amount of body fluid, mix the secretion with their legs, and skillfully plaster it on the injury of the plant (Kutsukake et al. 2019). The body cavity of soldier nymphs is full of large highly differentiated hemocytes that contain huge amounts of lipid droplets and phenoloxidase (PO). Upon release, these cells lyse and produce reactive quinones that crosslink lipids and various macromolecules to physically reinforce the clot and seal the breach. It is not known whether there are equivalent cells in pea aphids, but the different studies of the circulating hemocytes has not described such cells. Alternatively, they may form a specific tissue that has

not yet been identified. The identification of the source of PO in pea aphids will therefore be a topic in the future to study by which mechanism *Hd* and *Ri* regulate its expression and amount and therefore an important factor of humoral immunity.

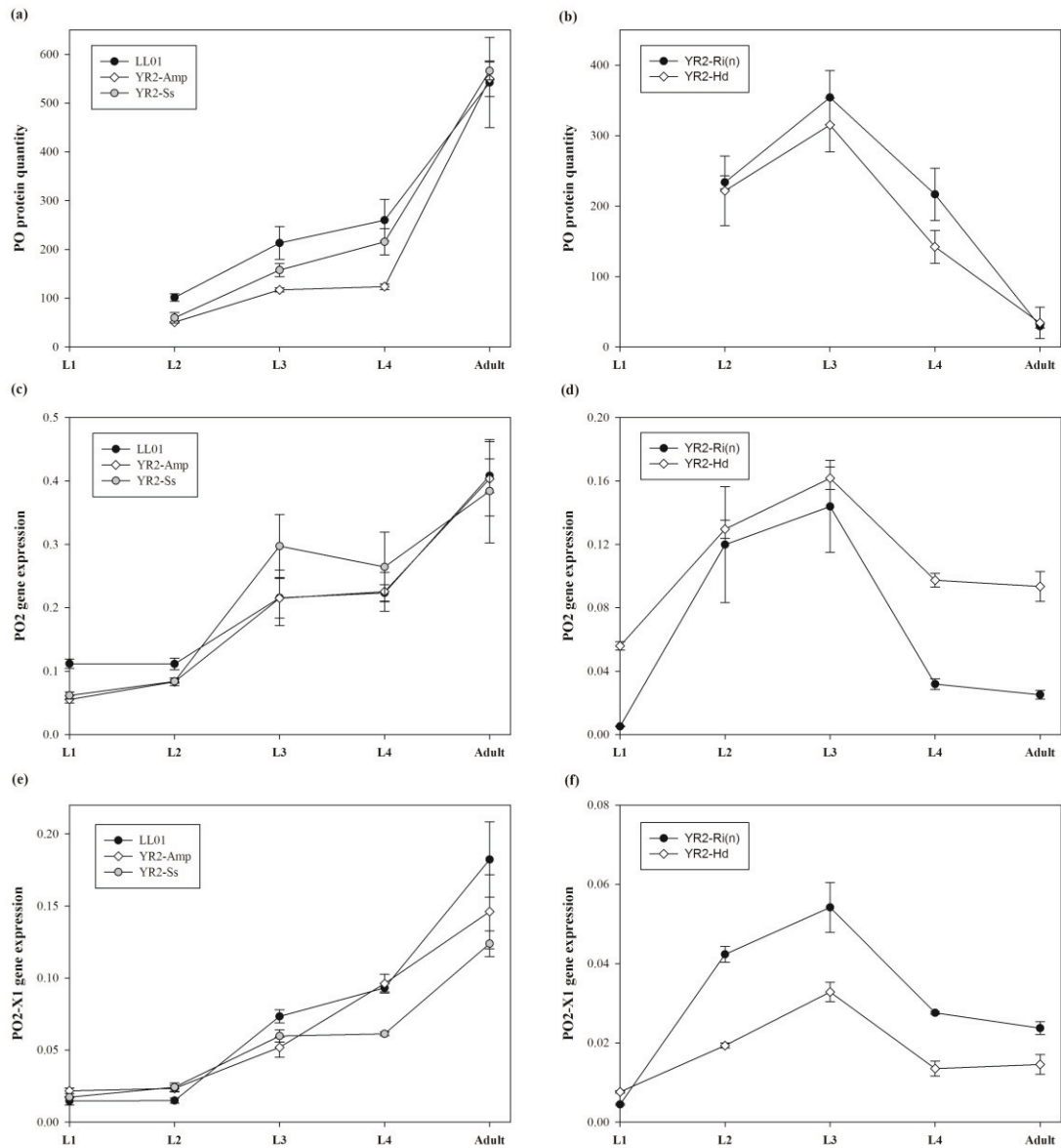


Fig. 28. Dynamics of the amount of PO protein and the expression of PO genes during the development of aphids. (a, b) PO amount of different development stages in aphid lines LL01, YR2-Amp and YR2-Ss (a) and in YR2-Ri(n) and YR2-Hd (b). For technical reason (low amount of protein extracted in L1), the amount of PO protein was relevant only from the L2 stage. Gene expression level for PO2 (c, d) and POA 3(e, f) in LL01, YR2-Amp and YR2-Ss (c, e) and YR2-Ri(n) and YR2-Hd (d, f) in function of the aphid stages. Three biological replicates for each line. For protein quantity, hemolymph from pools of 8 aphids to L2, 7 aphids to L3, 6 aphids to L4 and 5 aphids for adult were used. Pools of equivalent weight of 5 adult aphids were used for cDNA extraction to evaluate the gene expression level for every instar stage. The detailed methods for PO protein and gene expression are the same as described before (Luo et al. in Prep).

Table 10: Results of multi-comparisons analyzing throughout the development the different aphid lines, respectively. The ANOVA data of whole experiment were $F_{3,8} = 19.195$, $P = 0.001$ (LL01); $F_{3,8} = 491.995$, $P < 0.001$ (YR2-Amp); $F_{3,8} = 143.872$, $P < 0.001$ (YR2-Ss); $F_{3,8} = 97.087$, $P < 0.001$ (YR2-Ri(n)) and $F_{3,8} = 11.532$, $P = 0.003$ (YR2-Hd) for PO protein; $F_{4,10} = 27.099$, $P < 0.001$ (LL01); $F_{4,10} = 42.696$, $P < 0.001$ (YR2-Amp); $F_{4,10} = 22.550$, $P < 0.001$ (YR2-Ss); $F_{4,10} = 59.595$, $P < 0.001$ (YR2-Ri(n)); $F_{4,10} = 40.561$, $P < 0.001$ (YR2-Hd) for PO2 gene; $F_{4,10} = 89.462$, $P < 0.001$ (LL01); $F_{4,10} = 44.637$, $P < 0.001$ (YR2-Amp); $F_{4,10} = 116.146$, $P < 0.001$ (YR2-Ss); $F_{4,10} = 215.914$, $P < 0.001$ (YR2-Ri(n)); $F_{4,10} = 27.511$, $P < 0.001$ (YR2-Hd) for PO2-X1 gene.

Multi-comparisons	LL01	YR2-Amp	YR2-Ss	YR2-Ri(n)	YR2-Hd
	P-value (PO protein; PO2 gene; PO2-X1 gene)				
L1- L2	1.000; 0.999	0.197; 0.994	0.687; 0.066	<0.001; <0.001	<0.001; 0.007
L1- L3	0.012; <0.001	<0.001; 0.005	0.001; <0.001	<0.001; <0.001	<0.001; <0.001
L1- L4	0.006; <0.001	<0.001; <0.001	0.001; <0.001	<0.001; <0.001	0.006; 0.224
L1- Adult	<0.001; <0.001	<0.001; <0.001	<0.001; <0.001	0.001; <0.001	0.012; 0.124
L2- L3	0.045; 0.011; <0.001	<0.001; 0.002; 0.008	0.023; 0.003; <0.001	0.128; 0.874; 0.162	0.308; 0.030; 0.003
L2- L4	0.013; 0.006; <0.001	<0.001; 0.002; <0.001	0.001; 0.006; <0.001	0.917; 0.003; 0.007	0.430; 0.030; 0.227
L2- Adult	<0.001; <0.001; <0.001	<0.001; <0.001; <0.001	<0.001; 0.001; <0.001	<0.001; 0.001; 0.001	0.023; 0.015; 0.388
L3- L4	0.811; 0.989; 0.630	0.826; 0.999; 0.034	0.195; 0.982; 0.998	0.050; 0.001; <0.001	0.034; <0.001; <0.001
L3- Adult	0.012; 0.008; 0.003	<0.001; 0.030; 0.001	<0.001; 0.864; <0.001	<0.001; <0.001; <0.001	0.002; <0.001; <0.001
L4- Adult	0.040; 0.015; 0.022	<0.001; 0.045; 0.259	<0.001; 0.587; <0.001	<0.001; 0.858; 0.496	0.023; 0.991; 0.993



GENERAL DISCUSSION

My PhD work, in the field of host-parasite-microbe interactions, was based on the aphid-symbiont-parasitoid model. It focused on the effect of facultative symbiont species in modulating immunity and life-history traits of host aphids under normal or stressed conditions, in laboratory setting. Experiments that were carried out include: (a) the interaction between parasitoids and aphids and the role played by defensive symbiont and host plants; (b) the effects of common symbionts on the components of innate immunity and life parameters of host aphids under normal and different stressed conditions. Although this work cover different fields, they are linked in an ecological perspective: for example, the presence of symbionts that provide a protective phenotype to their hosts will decrease their mortality risk and increase their overall fitness, and under pressure, the proportion of individuals carrying defensive microorganisms may increase rapidly (Ford and King 2016; Hahn and Dheilly 2016). This and the fact that defensive microbes can shape the evolution of virulence of the pathogen (King and Bonsall 2017) can have an impact of the stability of the community in the field. Although some systems have been well studied, our knowledge of the diversity, distribution, mechanisms and ecological consequences of defensive symbioses is still limited. In aphids, these symbiotic effects would influence the ecology and evolution of their parasitoids and associated population-level processes (Fig. 29), as well as the biocontrol strategies put in place to fight this pest (Monticelli et al. 2019; Käch et al. 2018; Vorburger and Perlman 2018).

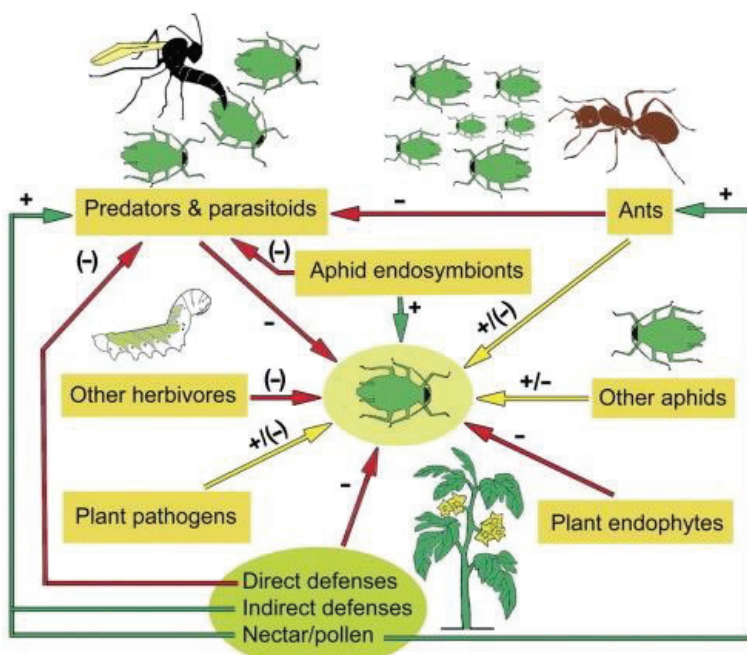


Fig. 29. The aphid networks. Aphid population growth and its impact on plant fitness are strongly influenced by interactions with other organisms, including plant pathogens, endophytes, aphid endosymbionts, predators, parasitoids, ants, and other herbivores. (From Goggin 2007)

Lucky or unlucky parasitoid choice



The concept of generalist and specialist is widely used in studies aimed at understanding the mechanisms of species evolution, particularly through specialization mechanisms. This concept of specialization is at the heart of the field of evolutionary ecology, behavioral ecology and community ecology (Rieseberg 2001; Kassen 2002; Loxdale et al. 2011). Central because the ecological characteristics of generalist and specialized species are at the base of the spatial distribution of species and the organization of food webs (Devictor et al. 2010). Indeed, natural selection favors more or less specialized strategies in species, according to an evolutionary compromise between the ability of a species to exploit a resource gradient and the performance on this resource (Levins 1968). Specialized strategies should benefit from relatively homogeneous environments in space and/or time, while general strategies should benefit from heterogeneous environments in space and/or time (Kassen 2002; Hooper et al. 2005; Ostergard and Ehrlen 2005). In order to classify a species as a specialist, intermediate generalist/specialist or a generalist, many aspects of their biology, ecology, and phylogeny must be considered. To Poulin and Mouillot (2003), the host range is a function of the number of hosts it can use and the relatedness of these hosts. Develop on this question Helmus et al. (2007) and Helmus and Ives (2012) proposed the use of phylogenetic species variability, which has been shown to be a more meaningful metric of the specialization of aphid parasitoids (Desneux et al. 2012). The description of the host range of parasitoids makes it possible to determine their host specificity and therefore their potential effectiveness as biological control agents (Heimpel and Mills 2017). However, ecological factors specific to each ecosystem can modify these host ranges and thus the position of parasitoids on the continuum between generalist and specialist species. Our work (publication 2) has identified the direct impacts of phylogenetic relationships on the theoretical host range of aphid parasitoids. In fact, specialized parasitoids on their hosts or their association with the host plant in hosts or their host-plant have attacked a large number of host species and differences have been observed mainly during their development. For example, the parasitoid *A. abdominalis* has a generalist behavior and attacks different host species but it only develops in phylogenetically related aphid species within the Dactynotina sub-tribe.

Factors that do not directly affect the host range of parasitoids modulate their success. This is the case of the presence in aphids of defensive symbionts (as observed in publication 1 and 2). We found that the presence of *H. defensa* conferred less resistance to generalized parasitoids (i.e. *Aphelinus abdominalis* and *Diaeretiella rapae*) than to the oligophagous parasitoid

Aphidius ervi (Oliver et al. 2003, 2010). New evidence has shown that defense symbionts confer protection only against the more specialized natural enemies and less against generalist ones (Asplen et al. 2014; Hrček et al. 2016; Kraft et al. 2017; Parker et al. 2013), suggesting the impact of defensive symbionts will be variable depending on the aphid-parasitoid system considered. When parasitoid oviposit in these suboptimal hosts this act as an “ecological trap” for parasitoids since they lose eggs in these hosts despite their negative effect on the success rate of parasitism. Their inability to discriminate these suboptimal hosts may lead to (i) reduced parasitism in potential host species (due to reduced eggs availability), (ii) drastically reduced parasitoid population in the environment, and (iii) under extreme conditions, their extinction. When individuals still emerge from these suboptimal hosts, they may suffer sublethal effects resulting in a significant decrease in their life history traits such as size (correlated with egg numbers) and lifespan (Godfray 1994).

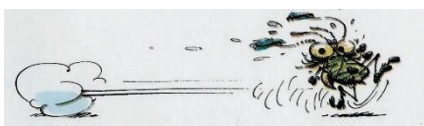
In Publication 1, we found that the presence of *R. insecticola* in *Sitobion avenae* could increase the rate of parasitism of one parasitoid species *Aphelinus asychis*, but not to another species *Aphidius gifuensis*. The presence of *R. insecticola* is not only detrimental to the aphid (more are parasitized and die mummified), but also to the parasitoid wasp whose success and weight of offspring are reduced. In this case, parasitizing infected aphids is therefore counterproductive for *A. asychis* since more energy must be invested to achieve parasitism and obtain the same final production of offspring. These data also suggest that the genotype of the aphid is important for the outcome of the interaction, an observation that may seem surprising and difficult to explain. One possibility is that infected aphids are less prone to defend themselves and are therefore more easily parasitized, but this does not explain why the other parasitoid did not perform better. In contrast, the emergence rate of *A. asychis* decreased due to higher mortality during development, suggesting a protective effect of *Regiella*. Although the protective phenotype is not entirely new for this symbiont (Vorburger et al. 2009a; Luo et al. 2017a), the increased attractiveness, the easiness of oviposition and the lethal effect on development of parasitoid larvae seems all to depend on the wasp species.

In both Publication 1 and 2, we found that wasps emerged from aphids infected by *H. defensa* or *R. insecticola* experienced a significantly higher mortality or deterioration (i.e. body weight). For *H. defensa* this is consistent with numerous studies showing an effect on the development of parasitoid larvae, possibly by bacterial toxins and those form by bacteriophage APSE (Degnan et al. 2009; Hansen et al. 2012; Oliver and Higashi 2019). *R. insecticola* also retains pathogenic characteristics and can produce similar toxins. However, in both cases, the effect may also result from a competition between bacteria and parasitoids for the use of metabolites,

as observed in *Drosophila* (Paredes et al. 2016), or by altering the overall physiology of the host in a detrimental way for the developing parasitoid larvae (i.e. eliminating the teratocytes). The effect of the symbiont on host immunity to fight the parasite could not be ruled out. Reports have also shown that aphid species such as *Aphis nerii* and *Brevicoryne brassicae* are able to sequester plant products such as cardenolide (*Asclepias*) and glucosinolate (cabbage), respectively (Jones et al. 2001; Desneux et al. 2009a), and these toxic allelochemical molecules have a huge impact on the survival of immature parasitoids (Mooney et al. 2008; Pratt et al. 2008; Desneux et al. 2009a; Kos et al. 2012). Interestingly, aphid feeding plants release volatile substances that attract parasitic wasps, while endosymbionts attenuate the systemic release of such volatiles by plants after an attack of aphids, as well as the recruitment of parasitic wasps therefore increasing the aphid fitness (Frago et al. 2017). It is therefore not unconceivable that *Regiella* may also help aphids to produce toxic metabolites from plant products to protect them from parasitoids or pathogens. However, the conferred protection is at cost because the infected aphids having survived the attack of *A. asychis* (but not *A. gifuensis*) have clearly reduced survival and fecundity compared to the control. Overall, these results confirm and corroborate previous studies showing that the cost/benefit of the presence of a facultative symbiont depends on the genotypes and the context (Feldhaar 2011; Cayetano et al. 2015; Hrček et al. 2016; Martinez et al. 2018).

Publication 1 and 2 contribute to understanding the host range of several parasitoid species, and extend the protective phenotypes to a new strain of *Regiella*. They also outline the role of the symbiont genotype x aphid genotype effect in the expression of this phenotype. Such knowledge can be used to more accurately predict the efficacy of the parasitoid by identifying (i) an effective parasitoid, (ii) the potential risks of introducing this parasitoid to non-target species, (iii) the factors hindering the establishment of a parasitoid in a given environment, and (iv) certain factors possibly reducing the ability of the parasitoid to control a pest population.

Of symbionts and stressors effects on aphid immunity and life parameters



Host defense against biotic stresses (e.g. parasitic wasps) relies primarily on the host immune system, which plays a key role in the evolution process of the host (Poirie and

Coustau 2011). The knowledge about the interactions between the immune system of aphids and their vertically transmitted-symbionts is limited, although new evidence suggests that symbionts may impact positively or negatively the immune system of insects, either through

metabolic actions or by still unknown molecular mechanisms (Chevalier et al. 2011; Weiss et al. 2011; Schmitz et al. 2012). Knowledge of the physiological components of the pea aphid's immune system is emerging. Its genome study showed a reduction in immune pathways (Gerardo et al. 2010) and a poor response to pathogens due to the absence of most antimicrobial peptides from known insects. We used the pea aphid to explore the effect of its three most common secondary symbionts on a key humoral component of the innate immune system of insects, the phenoloxidase (PO) (the speed-limiting enzyme in the melanization process) and its regulation, as well as on the aphid life-history traits under normal and different stress conditions. Recent research also shows that insect PPO has a relationship with neuronal activity, longevity, melanization of feces (phytophagous insects) and development.

In publication 3, we confirmed that both PO genes (PO2 and PO2-X1) are expressed in pea aphids (Gerardo et al. 2010; Xu et al. 2019), and our data suggest that the corresponding proteins are also synthesized and secreted in the hemolymph. The presence of PO in the hemolymph was first demonstrated using 2D gel and mass spectrometry, an analysis that also gave information on the major proteins of the aphid hemolymph, revealing that it carries several proteins from the *Buchnera* symbiont, such as GroEL (Fares et al. 2004), a bacterial chaperone present also in the saliva of the aphid and which elicits defense reactions in plants (Chaudhary et al. 2014). The hemolymph contained the bulk of the phenoloxidase present in aphids, with the rest of the aphid carcass accounting for less than 10% of the total aphid PO protein. We could not determine with certainty whether the two PO proteins were present in the hemolymph, since they have almost the same molecular weight and cannot be separated by electrophoresis. The peptides obtained by mass spectrometry matched the two sequences and our antibody theoretically recognizes both proteins. As the sequences of both PPO sequences retained the proteolytic site observed in the PO of many other insects (Lu et al. 2014) (Fig. 30).

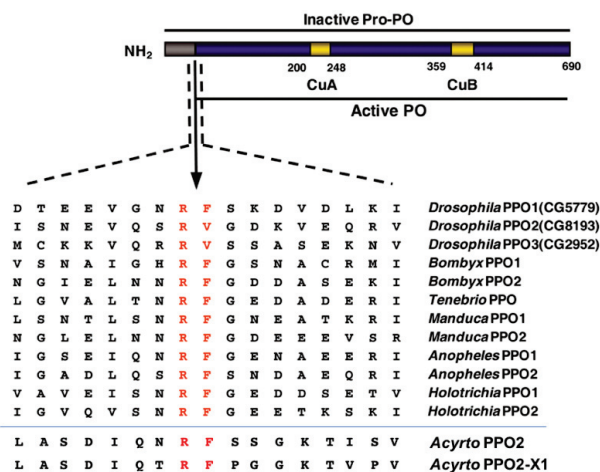


Fig. 30. The structural organization of Pro-PO members. The cleavage region (boxed) of *Drosophila* Pro-POs and other insect Pro-POs are compared to the same sequence region of pea aphid PPO2 and PPO2-X1. The cleavage site in Pro-PO is indicated by an arrow. CuA and CuB are the two Cu⁺⁺ binding site of the active PO. (From Nam et al. 2011).

We observed that although they have conserved the cleavage site, no N-terminal cleavage was required to activate the PPOs of aphids; this suggests that they are self-activated by a conformational change such as *D. melanogaster* PPO3 (Liu et al. 2012). The similarities with dmPPO3 go far beyond that, because the POs of *A. pisum* and dmPPO3 do not require alcohol treatment or copper addition to be active *in vitro* (Liu et al. 2012). We further tried to knockdown the two different POs by RNAi but unfortunately, we failed. In their data, Xu et al. (2019) that succeeded in this task, showed that elimination of PPO2 alone reduced the spontaneous PO activity in the aphid hemolymph, while extinction of the two POs suppressed it. Although not definitive, this support our data and strongly suggests that both proteins are produced in a circulating form and participate in the PO activity. It is difficult to believe that hemocytes contribute so little to PO, since in insects, different type of hemocytes produce PO (i.e, crystal cells in *Drosophila* (Rizki and Rizki 1959), oenocytoids in mosquito (Castillo et al. 2006) and lepidopterans (Liu et al. 2013)). In the study of Schmitz et al. (2012), 4% of adherent hemocytes had the capacity to produce melanin when incubated with DOPA, suggesting the presence of PO. With our antibody, we confirmed this proportion of PO positive adherent hemocytes (Fig. 31).

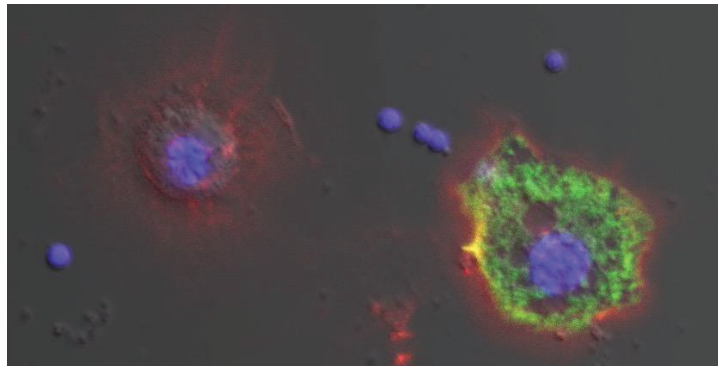


Fig. 31. Immunolocalization of PO in adherent aphid hemocytes. Adherent hemocytes (AH) were labelled with the anti-PO that was revealed with a green fluorescent secondary antibody. In reactive cells such the one in the right, PO was scattered throughout the cytoplasm. Not all cells were PO-positive as shown by the unlabeled cell at left, in fact only 4% of AH were reactive. Actin was stained by red fluorescent phalloidin and nucleus in blue. The blue free dots are either prohemocytes nucleus or *Buchnera* cells (Schmitz et al., unpublished)

Unlike the pea aphid, the body cavity of soldier nymphs *Nipponaphis monzeni*, a social aphid, contains large hemocytes filled with high amounts of lipid droplets and rich in PO, which are expelled during defensive behaviors to protect the aphid gall (Kutsukake et al. 2019). In their additional data, the authors indicated that *A. pisum* hemocytes are enriched in PO mRNA with respect to the whole body. However, there was no difference in the amount of POs mRNA between whole and bled aphids, suggesting that the majority of POs mRNA was not in the

hemocytes. We do not yet have an explanation for this difference apart from hemolymph collection methods (they get the hemolymph after cutting the aphid legs while we do a small ventral opening) and the quantification method (PCR versus RNAseq). However, in the laboratory, we have also done a RNAseq approach of the adherent hemocytes (granulocytes + plasmatocytes) and for the YR2 line, none of the POs are among the top expressed genes, but this analyze needs to be finalized (Cardinaud et al. unpublished). Thus, although our data suggest that adherent hemocytes of pea aphid are not the main source of PO protein, more studies will be required to definitively conclude, as some non-adhesive hemocytes, such as spherulocytes, rapidly lyse upon hemolymph collection (Schmitz et al 2012) and may also contain POs.

Our results showed that the presence of *H. defensa* or *R. insecticola* but not *S. symbiotica* could significantly decrease the PO levels through a decrease in gene expression, protein amount and protein activity (these three parameters being highly correlated). These effects were observed with two different aphid genetic backgrounds. The symbiont effects were visible at the level of genes expression, which were reduced by at least 50%, but maybe also at the proteins production level since the protein quantity was decreased by more than 80%. *H. defensa* and *R. insecticola*, but not *S. symbiotica*, could thus selectively affect the humoral immune system of pea aphids. The genomes of *R. insecticola* and *H. defensa* are divergent but share ~ 55% of their genes (Rollat-Farnier et al. 2015). The functions encoded by these shared genes include host cell toxicity and invasion capabilities (type 3 secretion systems and RTX toxins, which are pore-forming exotoxins (Degnan et al. 2010)). In contrast, the genome of *S. symbiotica* from pea aphid showed significant pseudogenization and inactivation of these pathogenesis genes (Burke and Moran 2011). We therefore hypothesize that some of the RTX toxins secreted by *H. defensa* and *R. insecticola*, and absent from *S. symbiotica*, could, by their cytolytic capacity (Linhartová et al. 2010), affect the production of PO, possibly by targeting the producer cells. In addition, we observed that these effects on PO resulted from a reduction in gene expression and protein synthesis during nymphal development of the aphid, particularly in stage 3, whereas the number of facultative symbiont cells seemed to increase strongly, probably colonizing cells and tissues. The cell/tissues producer of PO may be very sensible to these bacterial toxins.

Stress conditions (injection of inert latex beads, live Yeast or *E. coli*) induce a reduction in survival and an increase in reproduction in all lines, regardless of the basal level of PO. This is consistent with the hypothesis that fertility compensation is used as an effective stress response in organisms like aphids having a restricted immunity (Altincicek et al. 2008; Barribeau et al.

2010). Shortly after the stress (24h), the activity and amount of PO increased only in the YR2-Amp and -Ss lines (those with a higher basal PO level) and in response to yeast injection. Surprisingly, this was not correlated to gene expression since mRNAs quantity for both POs decreased 24 hours after infection. Since we have not detected any “stocks” of PO in these lines, the only possible explanation is that the PO mRNAs present are highly transcribed into PO proteins before they decline or that the expression of PO increases transiently very rapidly (a few hours) just after stress.

Adaptation to facultative symbionts

A recurring question is how the aphids handle the facultative symbiont and vice versa. We have shown using long established lines that some symbionts can reduce the immunity (cellular and humoral) of their host. To evaluate how long it could take to a symbiont to subdue the host immunity, we injected *S. symbiotica* and *H. defensa* into the YR-Amp line and used the PO as an immunity marker. The injection of both symbionts induced an immune response in YR2-Amp, like injection of yeast and to a lesser extent *E. coli* (see publication 3). After two generations, the amount of PO in the newly created lines has returned to its basal level and remained at that level for two or three generations. Subsequently, the amount of PO decreased significantly in the next generation in the presence of *H. defensa* to reach the long-term level of the YR2-Hd line. In addition, we also found that the fecundity and survival of newly infected lines with *H. defensa* decreased as they were in long-term established lines. This suggests a rapid adaptation between the aphid host and its secondary symbionts. It also shows that infection with *Hd* has a rapid and powerful effect not only on PO but on some other physiological parameters in a few generations. It may be interesting in the near future to analyze the other changes occurring during these two or three phases of adaptation observed using the PO as a marker; For example, the number of hemocytes, of bacteriocytes (and of *Buchnera* cells), of the symbiont cells in the case of *Hd*, and the tissue localization of the secondary symbionts. Indeed, the facultative symbionts must colonize certain bacteriocytes or sheath cells or induce their production. If these different phases are recovered, a study of gene expression of the host and maybe the symbiont at different times could be performed to analyze the different host pathways controlled by the establishment of a secondary symbiont.

CONCLUSION AND PERSPECTIVES



Our studies emphasize the role that facultative symbionts can have on the ecological network of aphids. We have shown that the defensive symbiont plays a role in protecting against the parasitoid attack, and the choice of an infected host has a fitness cost for parasitoids. Indeed, we have demonstrated that a defensive symbiont strain of *R. insecticola* modulates the outcome of host-parasitoid interactions (oviposition and success) in a wasp species-dependent manner, but with a fitness cost for the surviving host. It will be interesting in the future to transfer this particular *Ri* symbiont isolate in different aphid species and then analyze its phenotypic effects. Sequencing the genome of this isolate and comparing it with those of already characterized defensive and non-defensive *Ri* strains can also be scheduled.

We also demonstrate that the secondary symbionts impair their host immunity in a symbiont-dependent manner and, interestingly, that the most efficient symbiont is the one that has retained pathogenic traits. Although variation in PO did not have a significant effect on the outcome of the different stresses we tested, some variations observed could be significant under natural conditions. We demonstrate that this symbiotic effect on immunity begins with one of the stages of nymphal development of aphids, allowing further studies to be focused on this topic at this time, both for the host and for the symbiont. This effect on immunity was observed after a few generations when the pathogenic secondary symbiont was injected in an aphid, suggesting a rapid adaptation between symbionts and their host, mostly symbiont driven. In subsequent studies, it will be of interest to analyze the effect of secreted bacterial toxins on the development of the aphid immune system and determine whether they could impact the transcriptional machinery in host cells or destroy some cells involved in immunity (hemocytes, PO producing cells). In this regard, the origin of aphid hemocytes, including the existence of a hematopoietic organ, will be mandatory.

Although the beneficial nature of primary symbiosis for the insect host is widely demonstrated, the costs/benefits generated by the presence of facultative symbionts must be explored as they will impact the short- and long-term evolution of the symbiotic association. In recent years, the increasing number of studies of symbiotic interactions in mammalian models have brought to light the complexity and importance of the intestinal microbiome for Human health. Aphids provide a simplified and useful model for exploring how symbionts interact with their host, a field of research that will be a fascinating challenge for the future.



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APPENDICES

Publications

Effect of the endosymbiont *Regiella insecticola* on an aphid parasitoid

C Luo, L Monticelli, L Meng, D Li, J Fan, H Zhao, Z Hu

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Article

Effect of the endosymbiont *Regiella insecticola* on an aphid parasitoid

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With 2 figures

Abstract: The importance of secondary endosymbionts on the resistance to parasitoids is increasingly recognized. Compared with *Hamiltonella defensa*, our understanding of the role of *Regiella insecticola* in protecting hosts against parasitic wasps is not well documented. In this study, we conducted experiments to determine whether *R. insecticola* in *Sitobion avenae* (the English grain aphid) could confer resistance to a parasitoid, *Aphelinus asychis* Walker, a major natural enemy in aphid field populations. With the genetic background controlled using clones, our results showed that infected aphids were more likely to be parasitized by *A. asychis*. However, compared with the uninfected controls, the weight of emerged wasps within 24 hours was significantly lower from infected aphids, suggesting that *R. insecticola* negatively affected the growth of the developing parasitoid larvae, although no difference in emergence rate between the treatments and the control was detected. These results will help to increase understanding of the dynamics of host-parasite-symbiont interactions.

Keywords: Secondary endosymbiont, *S. avenae*, Parasitoid, Resistance

Intraspecific variation in facultative symbiont infection among native and exotic pest populations. Potential implications for biological control.

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Intraspecific variation in facultative symbiont infection among native and exotic pest populations: Potential implications for biological control

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ABSTRACT

Facultative bacterial symbionts can provide their host insects with protection from natural enemies. These symbionts are often found at low to intermediate frequencies among hosts in native populations, suggesting that fewer symbiont taxa (and their corresponding defensive properties) may be present in exotic populations, due to founder effects and drift in newly established populations. We tested this hypothesis by collecting aphid species from their exotic and native regions, and conducting diagnostic surveys for four facultative symbionts: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, and *Arsenophorus nasoniae*. We did not find fewer symbiont taxa in exotic host populations, but did find substantial intra- and interpopulation variation in symbiont infection. When we incorporated additional records from the literature, we found moderate support for the hypothesis, although few aphid species were sampled sufficiently to be conclusive. Finally, we tested whether laboratory colonies are prone to losing symbiont infection over time. We established four colonies of the cowpea aphid, *Aphis craccivora*, each initiated with a single aphid clone infected with *H. defensa*. Through repeated sampling, we found that all four colonies became uninfected over the course of one year. We suggest that symbiont surveys could aid importation biological control introductions by (1) establishing whether recently established exotic pest populations might have reduced symbiont complements and be particularly vulnerable to natural enemies, (2) providing clues on pest provenance, and (3) determining which native pest populations include the same defensive symbionts as the exotic target populations, as these may be the best sources for prospective agents. We also suggest that laboratory cultures of target and nontarget organisms be routinely monitored for symbiont composition, to ensure that laboratory trials produce field-relevant results.

1. Introduction

Invasive species represent ever-increasing ecological and economic threats (Hill et al., 2016). The globalization of human commerce provides extensive routes for accidental or deliberate movement of biological organisms into novel habitats across the planet (di Castri, 1989; Mack et al., 2000). While only a small percentage of these exotic species subsequently establish and spread within their new habitats (Williamson, 1996), those that do can be devastating. Pimentel et al. (2005) estimated that invasive species cost more than \$100 billion per year in the US, of which ~\$30 billion is attributed to exotic insects. Further ecological costs of invasive species include loss of biodiversity,

threat of native species extinction, and alteration or loss of environmental services (Kenis et al., 2008). Some populations of invasive insects have been effectively neutralized by eradication efforts (Myers et al., 2000) or through introductions of predators, parasites and pathogens from the source region of the invasive species (reviewed in Heimpel and Mills, 2017; Van Driesche and Bellows, 1996). However, many invasive species continue to exert major detrimental effects despite substantial investment in control efforts (Kovacs et al., 2010; Simonsen et al., 2008). Given the magnitude of the problems caused by invasive species, it is not surprising that major effort has been invested to better understand invasion dynamics and identify key factors that promote the establishment and spread of introduced species (Hill et al.,

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Résumé

Les symbioses sont omniprésentes dans la nature. Dans ces associations intimes et prolongées entre différents organismes, l'effet de l'expression des gènes d'un partenaire sur l'autre peut conduire à l'apparition de nouveaux phénotypes, un concept appelé "phénotype étendu". Ma thèse porte sur l'étude des interactions hôte-parasitoïde-symbiotes chez les pucerons, principalement le puceron du pois *Acyrtosiphon pisum*, devenu un modèle de par sa symbiose nutritionnelle obligatoire avec *Buchnera aphidicola* et facultative avec un ou plusieurs symbiotes, les plus courants étant *Hamiltonella defensa* (*Hd*), *Regiella insecticola* (*Ri*) et *Serratia symbiotica* (*Ss*). Le travail présenté aborde des aspects écologiques et physiologiques de la symbiose facultative chez les pucerons. Les pucerons sont des hôtes pour une communauté complexe de parasitoïdes qui s'inscrivent dans l'hypothèse Performance-Preference (PPH) selon laquelle les femelles vont préférentiellement pondre dans les hôtes qui maximisent la survie et les performances de leur progéniture. L'évaluation de la PPH permet de classer les parasitoïdes en termes de degré de spécialisation. J'ai participé à la détermination de la PPH de trois parasitoïdes (*Aphelinus abdominalis*, *Aphidius ervi* et *Diaeretiella rapae*) en utilisant 12 espèces de pucerons (6 Aphidini et 6 Macrosiphini) maintenus sur différentes plantes hôtes et dont le statut symbiotique était établi. *A. abdominalis* et *D. rapae* sont apparus comme des généralistes et *A. ervi* comme un spécialiste modéré. Toutes les espèces ont montré une faible sélectivité vis-à-vis de l'hôte quelle que soit la plante hôte ou le symbiote, mais le succès parasitaire était impacté par certains symbiotes. J'ai ensuite étudié l'effet du génotype de l'hôte, des génotypes (hôte x symbiote) sur la réussite des parasitoïdes sur des clones du puceron *Sitobion avenae* infectés artificiellement avec une souche protectrice de *Ri*. Les lignées infectées sont de meilleurs hôtes pour *Aphelinus asychis* mais pas *Aphidius gifuensis*, par rapport aux mêmes clones exempts d'infection. L'effet de *Ri* est donc dépendant de l'espèce parasitoïde, indiquant que le coût/bénéfice d'un symbiote dépend du contexte. Dans la seconde partie de thèse, je me suis concentré sur le système immunitaire de l'hôte en tant que facteur central dans l'établissement et l'évolution des interactions entre les organismes. L'annotation de différents génomes de pucerons montre une immunité réduite qui pourrait être due à leur adaptation à une vie symbiotique. Les hémocytes et l'activité phénoloxydase, deux composants immunitaires majeurs, ont été décrits chez le puceron. J'ai développé des outils moléculaires pour analyser l'expression de gènes codant pour les deux phénoloxydases (PO) d'*A. pisum* (PO2 et PO2-X1) et pour estimer leur quantité dans l'hémolymphe. J'ai utilisé des clones de différents fonds génétiques sans symbiote secondaire (LL01, YR2-Amp, T3-8V1-Amp) et les lignées naturelles ou artificielles YR2 ou T3-8V1 infectées par *Hd*, *Ri* ou *Ss*. J'ai démontré que : i) les deux gènes sont exprimés et que leurs produits sont présents sous une forme circulante dans l'hémolymphe, ii) l'expression des gènes, la quantité et l'activité de la PO sont fortement corrélées et dépendent du fond génétique de l'hôte et iii) ces trois marqueurs sont significativement diminués par la présence de *Hd* et de *Ri*. J'ai observé une corrélation entre l'impact des facteurs de stress sur les traits de vie du puceron et la présence de certains symbiotes (et donc la quantité de PO des pucerons), mais pas de corrélation avec la variation de PO après le stress. Ce travail montre donc une forte interaction entre la capacité immunitaire de l'hôte et le statut symbiotique du puceron, et il peut expliquer le succès ou l'échec de certains parasitoïdes qui ne sont pas hautement spécialisés pour l'hôte qu'ils attaquent.