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From epigenetics to bacterial symbionts – towards sustainable targets of aphid pest management

DISSERTATION

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"Insects nurture and protect us, sicken us, kill us. They bring us both joy and sorrow. They drive us from fear to hate, then to tolerance. At times they bring us up short to a realization of the way the world really is, and what we have to do to improve it. Their importance to human welfare transcends the grand battles we fight against them to manage them for our own ends. Most of us hate them, but some of us love them. Indeed, at times they even inspire us"

John J. McKelvey, Jr. 1975
Insects and Human Welfare

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DDT: Dichlordiphenyl-trichlorethan

BVL: German Federal Office of Consumer Protection and Food Safety

IP: insecticidal product

IPM: integrated pest management

GM: genetically modified

Bt toxin: modified toxins obtained from *Bacillus thuringiensis*

R genes: plant-derived resistance genes

dsRNA: double-stranded RNA

RNAi: RNA interference

EFSA: European Food Safety Authority

KAT: lysine acetyltransferases

KDAC: lysine deacetylases

GNAT: GCN5-related N-acetyltransferases

p300/CBP: p300/CREB-binding protein

MYST: MOZ/Ybf2/Sas2/Tip60

Sir: sirtuin

Epi-ML: epigenetic multiple ligand

SAHA: suberoylanilide hydroxamic acid

WCR: western corn rootworm

nt: nucleotides

GFP: green fluorescent protein

AMP: antimicrobial peptide

NDBP: non-disulfide bridged peptide

MIC: minimal inhibitory concentrations

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SUMMARY

Aphids are notorious insect pests that harm ornamentals and crops by transmitting viruses and feeding on phloem sap, thus causing substantial economic damage. The extensive use of chemical insecticides is still the dominant aphid control strategy. The consequences of this are obvious: rapidly evolving resistance, environmental pollution, and risks to non-target organisms. For the last two decades, traditional control measures have been under debate due to an increasing ecological awareness of the society. This ongoing scenario illustrates the necessity to develop alternative, novel strategies and methods for sustainable aphid management. This dissertation is devoted to aspects of aphid biology, which should be considered to refine the application of existing and novel insecticides. Underexplored target genes and enzymes that may assist in development of new substances for aphid control are treated and RNA interference (RNAi) as a promising alternative pest control strategy is discussed in detail.

The pea aphid (*Acyrthosiphon pisum*) is a well-known laboratory model organism to study the insect family Aphididae, insect-plant interactions, symbiosis with bacteria, aphid transmitted viruses and phenotypic plasticity. It was also the first hemipteran whose genome was fully sequenced. The intention of this thesis was to explore the role of epigenetic mechanisms in the ontogenesis of this aphid. Epigenetic modifications, e.g. the acetylation of histones, are heritable variations of DNA structure that affect gene expression or cellular functions without changes to the underlying DNA sequence. Such changes are regulated by the opposing activities of enzymes called histone acetyltransferases (KATs) and histone deacetylases (KDACs). These enzymes are essential in the regulation of numerous biological processes. In insects, the disruption of the tightly controlled equilibrium of acetylation and deacetylation of histones results in severely affected life-history traits, such as fecundity or longevity.

This thesis provides a comprehensive overview of histone acetylation/deacetylation enzymes present in the genome of *A. pisum*. Compared to other insects, an enlarged diversity of KATs and KDACs has been identified in the pea aphid. Epigenetic multiple ligand (3,5-Bis-(3,5-dibromo-4-hydroxybenzylidene)-tetrahydro-pyran-4-one) and suberoylanilide hydroxamic acid (*N*-Hydroxy-*N*'-phenyloctandiamide) were used for chemical inhibition of these enzymes, which negatively affected survival and reproduction and delayed the development of the aphids. These findings indicate that the epigenetic machinery is a promising target system for the development of novel aphid control substances. However, specific gene silencing of the KATs *kat6b, kat7, kat14*, and the KDAC *rpd3*, mediated by RNAi, revealed mild effects on life-history traits -mentioned above (**Kirfel et al., 2019**).

Conversely, the attenuation of the histone acetyltransferase p300/CBP severely reduced lifespan and the number of offspring of the aphids. A much shorter reproductive phase and more premature nymphs, which developed in abnormally structured ovaries, have been observed. These data confirmed the evolutionarily conserved function of p300/CBP known from

other insects during ontogenesis and indicated that this protein is an ideal target for RNAi-based aphid control (**Kirfel et al., 2020**).

It is well known, that aphids harbor the obligate bacterial symbiont *Buchnera aphidicola*, which is localized in a specialized organ (bacteriome), thus enabling the host to survive on a nutritionally poor phloem-sap diet by providing essential amino acids. Notably, aphids can live in symbiosis with a number of other, facultative symbionts (e.g. *Serratia symbiotica*, *Hamiltonella defensa*, and *Regiella insecticola*), facilitating adaptations to biotic and abiotic stress. In this context the question arose whether the symbiont *S. symbiotica*, which is present in the aphid line used in this study, has an effect on host fitness. In particular, the effect on the susceptibility of the aphids to different insecticidal compounds was investigated. Surprisingly, the facultative symbiont *S. symbiotica* was found to significantly impair the aphids' fitness and the ability of *A. pisum* to tolerate different classes of insecticides, although it does provide benefits in certain circumstances (**Skaljac et al., 2018)**.

Antimicrobial peptides (AMPs), key players of the insect's innate immune system, are short proteins with antimicrobial activity. During their long evolutionary history, aphids have lost many genes encoding AMPs, most likely because AMPs would harm their bacterial symbionts. The venoms of animals preying on insects contain molecules, including AMPs, which are highly efficient in targeting insects. Consequently, venom-derived scorpion AMPs, which are orally delivered to aphids, reduced their survival, reproduction and the density of bacterial symbionts. The AMPs compromised the bacteriome but also the aphid directly, holding the potential to be developed as bioinsecticides to replace or complement conventional insecticides for aphid control. (**Luna-Ramirez et al., 2017**).

ZUSAMMENFASSUNG

Blattläuse ernähren sich vom Phloem und übertragen Pflanzenpathogene, schädigen so Zier- und Nutzpflanzen, und verursachen folglich schweren wirtschaftlichen Schaden. Zur ihrer Bekämpfung werden seit Jahrzehnten vorrangig chemisch-synthetische Insektizide verwendet. Deren Einsatz ist jedoch mit nachhaltigen Folgeschäden verbunden: zu nennen sind vor allem die rasche Entwicklung stabiler Resistenzen, Umweltverschmutzung, und mögliche Risiken für Nicht-Zielorganismen. Bedingt durch das sich in den vergangenen beiden Jahrzehnten immer stärker entwickelnde Umweltbewusstsein der Bevölkerung, werden rein chemische Maßnahmen der Schädlingsbekämpfung zu Recht hinterfragt und die Entwicklung neuartiger, nachhaltiger Methoden eingefordert. Die vorliegende Dissertation beleuchtet daher solche Aspekte der Blattlausbiologie, die genutzt werden könnten, um den Einsatz bereits vorhandener Insektizide zu optimieren und neuartige Strategien zur Bekämpfung von Blattläusen zu entwickeln. Hierbei werden bislang wenig beachtete Zielgene und -enzyme sowie der Einsatz der RNAi-Technik als vielversprechende Alternativen detailliert betrachtet.

Die Erbsenblattlaus (*Acyrthosiphon pisum*) ist ein bewährter Modellorganismus zur Untersuchung von Röhrenblattläusen (Familie: Aphididae) und deren phänotypischen Plastizität, zum Studium von Interaktionen zwischen Pflanzen und Insekten, von Insekten-Bakterien-Symbiosen als auch von Insekten als Vektoren für Pflanzenviren. Die Erbsenblattlaus war überdies das erste Insekt aus der Familie Hemiptera, dessen Genom vollständig sequenziert worden ist. In der vorliegenden Dissertation wurde vorrangig die Bedeutung epigenetischer Mechanismen für die Ontogenese der Blattläuse untersucht. Epigenetische Modifikationen sind ererbte Variationen einer Genexpression oder zellulärer Funktionen, die jedoch ohne Veränderung der zugrundeliegenden DNA-Sequenzen entstehen. Solche Änderungen werden u.a. hervorgerufen durch das Wechselspiel von Histon-Acetyltransferasen (KATs) und Histon-Deacetylasen (KADCs). Diese Enzyme sind essentiell für die Regulation zahlreicher biologischer Prozesse. In Insekten konnte gezeigt werden, dass eine Störung des streng kontrollierten Gleichgewichts von Acetylierungs- und Deacetylierungsprozesse zu beträchtlichen Veränderungen in der Lebensdauer und Fruchtbarkeit führt.

Diese Arbeit bietet einen umfassenden Überblick über die im Genom von *A. pisum* codierten KATs und KDACs. Verglichen mit anderen Insekten, wurde in Erbsenblattläusen ein wesentlich breiteres Spektrum dieser Enzyme identifiziert. Der Einsatz spezifischer Inhibitoren (3,5-Bis-(3,5-dibromo-4-hydroxybenzylidene)-tetrahydro-pyran-4-one [= Epigenetic Multiple Ligand] gegen KATs; *N*-Hydroxy-*N*'-phenyloctandiamide – gegen KDACs) verkürzte die Lebensdauer, reduzierte die Nachkommenschaft und verzögerte die Entwicklung von *A. pisum*. Damit konnte gezeigt werden, dass die epigenetische Regulation ein vielversprechendes Zielsystem für die Entwicklung neuartiger insektizider Substanzen darstellt. Jedoch beeinflusste

die RNA-Interferenz spezifischer KATs und KDACs (*kat6b, kat7, kat14, rpd3*) die zuvor beschriebenen Fitnessparameter nur geringfügig (**Kirfel et al., 2019**). Wurde hingegen die Histon-Acetyltransferase p300/CBP spezifisch gehemmt, führte dies zu einer drastischen Verkürzung von Lebensdauer und Reproduktionsphase. Außerdem wurden nur wenige lebensfähige, dafür aber viele unterentwickelte Nymphen beobachtet, die sich in missgebildeten Ovarien entwickelten. Diese Daten bestätigen die Vermutung einer evolutionär konservierten Funktion von p300/CBP in der Ontogenese, wie sie bereits für andere Insekten beschrieben ist. Daher ist dieses Protein ein idealer Kandidat zur RNAi-basierten Bekämpfung von Röhrenblattläusen (**Kirfel et al., 2020**).

A. pisum lebt in Symbiose mit dem Bakterium *Buchnera aphidicola*. Dieser obligate Symbiont besiedelt ein spezialisiertes Organ, das sogenannte Bakteriom. Er ermöglicht es den Blattläusen, auf ihrer extrem nährstoffarmen Kost zu überleben, indem er ihnen essentielle Aminosäuren zur Verfügung stellt. Darüber hinaus können Blattläuse andere, fakultative Symbionten beherbergen (z.B. *Serratia symbiotica*, *Hamiltonella defensa*, oder *Regiella insecticola*), die u.a. Anpassungen an biotischen und abiotischen Stress erleichtern. In den dieser Arbeit zugrundeliegenden Experimenten wurden die Auswirkungen der Anwesenheit von *S. symbiotica* auf die Überlebensfähigkeit der Wirte und deren Empfindlichkeit gegenüber verschiedenen Insektiziden untersucht. Zwar profitiert *A. pisum* unter gewissen Umständen von der Anwesenheit des fakultativen Symbionten, andererseits werden dessen Fitness und seine Fähigkeit, verschiedene Klassen von Insektiziden zu tolerieren, erheblich beeinträchtigt (**Skaljac et al., 2018**).

Antimikrobielle Peptide (AMPs) spielen eine Schlüsselrolle im angeborenen Immunsystem von Insekten. Es handelt sich bei AMPs um kleine Proteine mit antimikrobieller Aktivität. Blattläuse haben im Laufe ihrer Evolution viele AMP-kodierende Gene verloren. Daher liegt die Vermutung nahe, dass AMPs ihren bakteriellen Symbionten erheblich schaden würden. Die Gifte solcher Tiere, denen Insekten als Beute dienen, sollten AMPs enthalten, die insbesondere zur Blattlausbekämpfung geeignet sein könnten. Es konnte gezeigt werden, dass die Verfütterung solcher AMPs die Überlebensfähigkeit und die Vermehrung der Blattläuse sowie die Konzentration der bakteriellen Symbionten reduzierte. Die AMPs scheinen dabei nicht nur das Bakteriom zu beeinflussen, sondern auch unmittelbare Auswirkungen auf die Blattläuse selbst auszuüben. Diese Ergebnisse veranschaulichen das beachtliche Potential von AMPs auf der Suche nach biologischen Bekämpfungsmöglichkeiten. Daher stellen AMPs eine interessante Alternative dar, um konventionelle Insektizide ganz oder zumindest teilweise zu ersetzen (**Luna-Ramirez et al., 2017**).

1. INTRODUCTION

1.1 THE BIOLOGY OF APHIDS – THE PEA APHID *ACYRTHOSIPHON PISUM*

Aphids (Aphidoidea) are small, hemimetabolous, plant phloem sucking insects, belonging to the order Hemiptera. The majority of all aphid species are found in temperate regions of the northern hemisphere. Together with psyllids (Psylloidae), whiteflies (Aleyrodoidea) and scale insects (Coccoidea), Aphidoidea comprise the Hemipteran subtaxon Sternorrhyncha (Fig. 1A) [1]. As common for the insect order Aphidoidea, the mouthparts of the insects are transformed into a sucking apparatus, which is located between the coxa of the first walking legs, thus enabling aphids to feed on phloem sap. Their fairly uniform morphology is further characterized by a soft, oval-shaped body ensuing from a broadly fused head, thorax and abdomen [2]. Conversely, the development and the lifecycles of aphids largely vary among species and include alternations of sexual and asexual generations, with oviparous or viviparous, as well as alate and apterous individuals (Fig. 1B) [3].

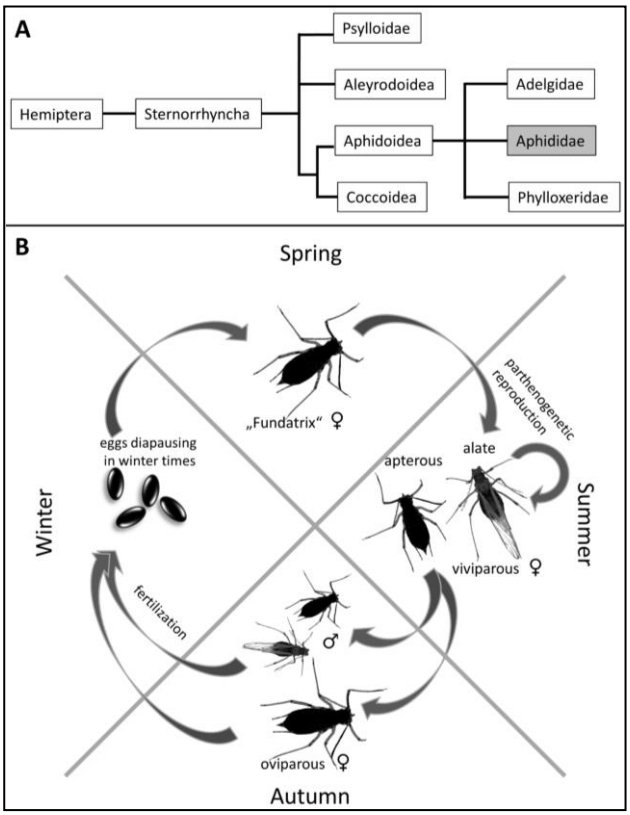

Figure 1. (A) Taxonomic origin of aphids [3]. (B) Life cycle of the pea aphid

Although some aphids can feed on a broad range of host plant species and are therefore considered generalists, about 99% of all Aphidoidea are specialists, associated with a defined host plant or a very limited number of closely related plant species. Some of these aphid species switch between two (specific) host plants, usually depending on seasonal changes [4]. The Aphidoidea can be further divided into three families, the Adelgidae (55 species), the Phylloxeridae (80 species), and the Aphididae. The latter is by far the largest subgroup, comprising ten recently described monophyletic families, which encompass over 4,400 species [3,5–7]. The exact phylogenetic resolution of these groups has been a matter of debate since their first description, and various correlations have been proposed [6–10]. However, the insect family Aphididae is clearly characterized by their member's life cycles that encompass viviparous generations, and the presence of the obligatory bacterial symbiont *Buchnera aphidicola* [11]. This symbiont is located in specialized cells (bacteriocytes) within an organ called the 'bacteriome' [12]. *B. aphidicola* provides the insect with essential amino acids

that allow the aphids to survive on their nutritionally poor phloem sap diet. Inversely, the aphids take over the biosynthesis of cell-surface components, regulators, and cell defense genes for and from the bacteria [13,14]. *B. aphidicola* is exclusively transmitted vertically to the offspring during embryogenesis. The bacteria are exocytosed from parental bacteriocytes in close proximity to the embryos [15] and subsequently endocytosed into the newly developing aphid [7,16]. This insect-bacteria symbiosis in aphids was first discovered and studied in the model organism *Acyrthosiphon pisum*, the pea aphid, a member of the aphid subfamily Macrosiphini (Hemiptera: Aphidoidea: Aphididae) [14]. Along with symbiosis, the pea aphid was taken as an archetype to study the Aphididae insect group as well as insect-plant interactions. Notably, *A. pisum* was the first Aphididae with a sequenced and annotated genome [14]. It is further used to explore phenotypic polyphenism, a phenomenon that describes the ability of an organism to translate environmental cues into distinct, reproducible phenotypic varieties, originated from a single genotype [14,17]. Several of these polyphenic forms are part of the complex life cycle of the pea aphid (Fig. 1B). Starting in spring, a wingless fundatrix hatches from an overwintering egg. The fundatrix reproduces asexually and viviparously, giving birth to up to ~120 winged or wingless female nymphs [18,19]. Wing development depends on the environmental conditions of the mothers and includes colony density, host plant quality, the presence of predators or parasites, etc. [20–28]. The nymphs go through four nymphal stages until they molt into adulthood and start to reproduce correspondingly [29]. In fall, shortening of the photoperiod finally triggers suppression of juvenile hormone production and induces changes in embryonic development. This results in the occurrence of one generation of wingless, sexual, and oviparous females as well as alate and apterous males [30–34]. Upon mating, the females lay inseminated, cold-resistant eggs, which undergo an embryonic diapause with an extraordinarily decelerated development during winter times [35]. Approximately 100 days later, asexual females hatch from these eggs and the life cycle starts over again (Fig. 1B) [36,37]. The monoecious pea aphids gain a body length of up to 5 mm, and its body color is pale green or pink (color polymorphism) with red eyes [38]. It is a major pest of peas and alfalfa, but can be found worldwide in a temperate climate, feeding on about 20 plant genera in the family Fabaceae, including *Medicago*, *Trifolium*, or *Lotus* [39].

1.2 ABOUT INSECT PEST CONTROL IN GENERAL AND APHID CONTROL IN PARTICULAR

1.2.1 INSECT PEST CONTROL – FROM PAST INNOVATIONS TO PRESENT PROBLEMS

Insects harbor the highest biodiversity of all eukaryotic groups in the animal kingdom [40]. They inhabit every single environmental niche on Earth (except the oceanic benthic zone) and are fundamental for all terrestrial ecosystems [8]. They are pollinators, dispensers, and they recycle nutrients. Insects maintain soil structure and fertility, control other organisms, and are a food source for other taxa themselves [41]. Regardless of their importance, some insects

became a threat to human health, or to the human food supply, by being food and feed competitors. Thus, the need for crop protection to prevent yield losses might be as old as crop domestication itself. First records of substances used to fight pest organisms date back to the old Sumerans, who used fumigated sulfur to fight insects or mites. Since 1000 B.C., salts of mercury, arsenic, cryolites or borax were used to control insect pests [42,43]. Later, organic preparations, such as ground *Tanacetum cinerariifolium* or tobacco were used as insecticides all over the world [44]. About 200 years ago, carbon disulfide and hydrocyanic acid expanded the range of compounds used as fumigants for pest control [45]. However, it was not until the 1930s, that the first synthetic compound with decided insecticidal activity was discovered. Dichlordiphenyl-trichlorethan (DDT), synthesized 1874 by Othmar Zeidler, revealed its insecticidal properties to Paul Müller in 1939 [43]. DDT was used repeatedly in public health programs to fight vector insects (and is still used occasionally) and probably became the best-known and most-expended chemical insecticide ever to prevent disease [46]. It was due to DDT that the elimination of endemic malaria from entire Europe was officially achieved in 1975. Its story of success ends, when growing concerns regarding the environmental persistence, fat solubility and, consequentially, the accumulation of DDT in non-target organisms, led to bans of DDT in most countries [44]. Additionally, more insecticide classes were discovered since the 1940s, such as organophosphates, pyrethroids, or carbamates. They increasingly replaced DDT as they were considered to be less toxic and stable in the environment [43,44,47]. Currently, the German Federal Office of Consumer Protection and Food Safety (BVL) reports 279 insecticides for crop protection [48]. Such products contain a total of 45 approved active substances [49]. In 2017, total sales of insecticides in Germany exceeded 850 metric tons of active substances [50]. Today, insect pest control products are considered one of the most important inventions for the rapidly growing human population. They improve human health by controlling vector insects, and secure food and feed supply by substantially decreasing crop loss [51]. Despite that, the discussion about problems associated with pest control and plant protection products continues. The concerns about the use of insecticides for crop protection involve their effects on non-target organisms including beneficial insects, bats, birds, worms, microorganisms, fish, etc. It is suggested that the extensive use of insecticides is one reason for the general decline in insect abundance and diversity, found recently [55]. Additional concerns include the stability and toxicity of the insecticides and/or their residues, (as well as safeners, co-formulants, adjuvants, synergists) to humans or livestock and the risk of eutrophication of the environment [54,56–62]. Perhaps the most important issue of insecticides is the dramatic increase of resistance, taking place in all kinds of agriculturally important pests [63–66] (Tab. 2). There is evidence that upon the introduction of every new pesticide group, cases of resistance occurred in various key pest species within 2 to 20 years [67].

Table 1. A selection of insecticidal products (IP) and strategies including advantages and disadvantages of these methods (according to Serazetdinova, 2019 [52]; Barzmann et al., 2015 [53], Keulemans et al., 2019 [54], the IPM Guides of the European Commission and the FDA)

IP/Strategy	Advantages	Disadvantages	Description
Synthetic Insecticides	• Highly effective • Easy to use • Scalable	• Bioaccumulation • Can be toxic to humans or non-target organisms • In most cases unspecific • Development of resistances • Environmental persistence • Demanding regulation	Synthetic substances with insecticidal activity
Biological insecticides	• Degradable • Easy to use • Can be less toxic than synthetic PPPs to humans and non-target organisms • Less demanding regulation • socially accepted • Can be specific • Scalable	• Can be toxic to humans • In most cases unspecific • Development of resistances • Can be less effective than synthetic PPPs, • Risk of eutrophication and acidification of land	A substance with an insecticidal or repellant activity which is of biological origin (animals, plants, bacteria) including extracts, pheromones or microorganisms
Mechanical control	• Free of chemicals or toxic substances	• Time-consuming • Inefficient • Often not suitable for large scale farming	Removal of pests by mechanical actions (e.g. shaking, picking) or protection through physical barriers (nets)
Crop rotation	• Easy to establish • Beneficial for biodiversity at the farm	• Highly dependent on biological context (e.g. present pests) • Reduces pest infestation, but cannot reduce crop-loss after infestation	Serial planting of crops, which are susceptible to different types of pests, thus, disrupting the pest's lifecycle
Biological control	• Reduces synthetic PPP usage • Increases biodiversity • Can be highly efficient • Pest control effect can be persistent • Resistances are unlikely • Can be specific • Easy to use	• Best suitable for closed environments (greenhouses) • Pest control effect can be delayed based on the reproduction time of biological control organism • Availability of control organism has to be assured	Application of natural predators or parasites including bacteria, nematodes, fungi, and viruses
Resistant cultivars	• Reduces chemical intake • an be designed specifically against target organisms	• Crop breeding is time-consuming • GMO use limited due regulation • Great potential to raise the resistance of pests	Use of crop strains, resistant to one or more particular pest-species
Smart farming	• Free of, or dramatically reducing pesticide use	• Very expensive • Technically demanding	Using sensors, cameras, unmanned (aerial) vehicles, high-tech monitoring and information technology to predict outbreaks of diseases, pests, etc.

At least one case of relevant field-evolved insecticide resistance has been confirmed in over 580 different insect species. Generally, four major types of resistance mechanisms are distinguished (Tab. 2) [68–70]. Moreover, the costs for development and registration of a new synthetic insecticide have exploded during the last two decades. Currently, they exceed 286 million US$ per insecticide during an average 12-year period [71].Growing environmental awareness, rising resistance issues and economic considerations led to the (re-)discovery, development and implementation of alternative plant protection products and strategies aside from chemical insecticides (Tab. 1). Now, there is an ongoing and accelerating transformation of pest control. One long-known but newly favored strategy is integrated pest management (IPM). It encompasses classical insecticides, but also novel or reconsidered products and

methods, including bioinsecticides, the use or extension of refugee areas, biological pest management, “smart farming”, and others, aiming to balance sociological desires, ecological needs, and economical expectations [53,54].

Table 2 Mechanisms of resistance (see also The Reflection and Research Network on Pesticide Resistance (R4P) 2016 [70])

Mechanism of resistance	Description
Target-site resistance	Genetic modification of the insecticidal target site eliminates or reduces the insecticidal effect
Penetration resistance	The permeability of the insects' cuticle is diminished in resistant insects
Metabolic resistance	Enhanced detoxification of a certain insecticide using increased levels of metabolic enzymes
Behavioral resistance	Active avoidance of the insecticide by the insect

1.2.2 APHID PESTS AND MANAGEMENT STRATEGIES

Aphids feed on phloem sap, severely damaging all kinds of plants, from crops in agriculture to ornamentals in horticulture [39]. This damage occurs directly through feeding in highly infested plants, but aphids are also vectors for a considerable amount of important plant pathogens, especially viruses [72]. As an indirect effect of sap-feeding, the aphids secrete large amounts of honeydew (a sugar-rich liquid) that covers all parts of the plants [73]. Subsequent, devastating fungal growth inhibits photosynthesis, reduces crop yields and decreases the market value of contaminated fruits and other field products [74,75]. Thus, aphids may cause yield losses worth billions of dollars each year [76,77]. The most prevalent practice to control aphids is the use of chemical insecticides including carbamates, organophosphates, pyrethroids, neonicotinoids and others [64,78]. Evidently, as a result of such a broad use of insecticides, a rapid selection for aphid resistance transpired. For example, the green peach aphid (*Myzus persicae*) has developed various resistance mechanisms (Tab. 2) to more than 70 synthetic compounds, and it is considered one of the most resistant insect species [79–81]. For modern aphid management, predictive models, which use parameters like rainfall, temperature and other climate conditions as well as publicly available trap catch data, have been developed and adapted to forecast aphid spread and aphid population development. These models help to optimize seeding time and to reduce the use of pesticides, thus providing a substantial benefit for growers and the environment [74,82].

Another method to prevent or delay the use of chemicals for aphid management is to engage in biological control using predators, parasitoids and microbial pathogens. Aphid parasitoids such as *Aphidius* spp. and *Aphelinus* spp., or aphidophagous organisms, including ladybirds, hoverflies and lacewings, have been established to decrease aphid abundance to a tolerable level in the field [74,83]. Still, biological control faces some challenges (e. g., potency and speed of effect) that have to be considered carefully [84–86].

A third strategy to restrict the aphid load on crops is the use of resistant plant varieties [87]. Such resistant cultivars can occur naturally, through breeding activities or are the result of genetic modification [88]. Genetically modified (GM) plants can be designed to deliver or release bioactive molecules for suppression of aphid population either by killing or by repelling the insect. A multitude of such bioactive molecules have been suggested, including protease

inhibitors, lectins, neurotoxins, modified toxins obtained from *Bacillus thuringiensis* (Bt toxin), aphid alarm pheromones or plant-derived resistance genes (R genes). Moreover, the use of double-stranded RNAs (dsRNAs) to inhibit essential aphid proteins through RNA interference (RNAi) has been proposed as a promising tool for aphid management [89]. Genetically modified pest-resistant crops, e.g. corn that produce multiple insecticidal Bt toxins, are already commercially available (e.g. YieldGuard™ from Monsanto or Herculex I® from Corteva Agriscience). However, no aphid-specific GM plants have entered the market yet and the future will show whether this is scientifically and economically feasible [90]. All over the world, more or less restrictive regulations are in place for GM crops. Especially the EU has very strict rules for the registration and cultivation of genetically modified plants. Many EU countries have even banned such plants completely. From a technical perspective, delivering bioactive molecules *in planta* entails a complex and long-lasting development of the transgenic plant. After ingestion, such compounds have to overcome gut defense mechanisms, target the gut directly, or have to access to target sides passing the gut epithelium. To circumvent such difficulties, non-plant strategies for topical application are now under investigation. The most obvious way is the direct application of the (formulated) bioactive molecules in sprays or powders. Additionally, recombinant viruses [91], (symbiotic) bacteria [92–95], fungi or microalgae are under discussion to be used as vectors to deliver the bioactive substances into the insects [96-99]. Next to spray-induced pest control formulations, additional exogenous application techniques are possible such as root or seed soaking, trunk injection, petiole absorption, or mechanical inoculation [98].

1.3 AIMS OF THE THESIS

The aim of this thesis was to shed light on different aspects of the aphids' biology that can be used to develop insecticidal compounds or strategies for aphid pest management apart from the traditional insecticides. Therefore, this thesis assessed the role of epigenetic modifications in aphids, or more precisely the importance of histone acetylation/deacetylation enzymes (KATs/KDACs) in the life history traits of the pea aphid. In the first step, a comprehensive overview of KAT and KDAC genes were supposed to be identified in the aphid genome. Then, it elucidated if these enzymes can be inhibited by chemical compounds such as Epi-ML (inhibition of KAT enzymes) and SAHA (inhibition of KDAC enzymes), and how such inhibition might affect various life-history traits of aphids. Additionally, it was intended to establish a robust RNAi protocol in *A. pisum*. Since achieving a high RNAi efficiency in aphids is known to be very challenging [100–103], the aim was to demonstrate that the pea aphid strain used herein is susceptible to RNAi. Subsequently, RNAi could be used for the specific attenuation of all previously identified KAT or KDAC genes. Doing this, the thesis aimed at the identification of target genes related to the histone acetylation apparatus, whose disruption reveals a major impact on the life-history traits of aphids. Thus, this thesis intended to find a

potent target gene that is suitable to be incorporated in RNAi-based pest control strategies for aphids. In addition, these experiments may uncover unknown aspects and new influencing variables of the aphid's RNAi machinery that cause the previously mentioned imponderability of RNAi. The findings may help to assess, whether RNAi is a reasonable and highly specific aphid control method.

While focusing on epigenetic traits and their potential as new targets for aphid control, this thesis additionally examines the influence of the facultative bacterial symbiont *S. symbiotica* on the life history traits of *A. pisum.* That included a closer look at the hypothesis that bacterial symbionts are crucial factors of insect tolerance against chemical insecticides and the idea that they may act as efficient targets for insect control agents itself. This information can help to optimize the use of artificial pesticides in the field. Finally, this thesis provides evidence that certain AMPs isolated from the venom glands of scorpions hold the potential to be developed as sustainable biopesticides.

2. MODERN ASPECTS OF APHID MANAGEMENT

"It is said that if you know your enemies and know yourself, you will not be imperiled in a hundred battles" – Sun Tzu, The Art of War

For the development of future insect pest control strategies, we have to consider numerous requirements. Such strategies have to be sustainable and ecologically friendly, they must not be a threat to public health, insect resistance has to be prevented, etc. (European Directive EC91/414, [63]). The Eurobarometer for food safety, commissioned by the European Food Safety Authority (EFSA) in 2019, revealed that people in the EU are concerned most about 'antibiotic, hormone and steroid residues in meat' (44%) followed by 'pesticide residues in food' (39%) [104]. Aphid management relies primarily on chemical insecticides. Due to the increasing demand in Europe, especially in Germany, to decrease the usage of chemical pesticides on cultivated land [105], there is a need to reconsider the traditional use of these substances. In the following chapters, aspects of aphid biology and life cycle will be discussed that offer new ways and strategies to refine or reduce the amount of currently used synthetic insecticides. It is obvious that novel targets and approaches are urgently required for sustainable control of aphids in the field.

2.1 THE EPIGENETIC MACHINERY – THE IMPORTANCE OF HISTONE ACETYLATION IN APHIDS

Ontogenesis requires a complex interaction of differentially expressed, controlled and regulated genes and their products, in order to convert a genotype into a phenotype. In some insects, such as aphids, this network appears to be even more complex, since multiple, distinct phenotypes may originate from a single genotype. This phenomenon is currently referred to as polyphenism. Such exceptional and heritable traits are thought to be environmentally triggered. Thus, their underlying molecular mechanisms need to be epigenetically controlled instead of being adjusted on the DNA sequence level. One possibility to regulate gene expression by means of epigenetic alternation is the chemical modification of chromatin residues. Subsequent changes in chromatin formation result in open euchromatin or closed heterochromatin. This modifies accessibility of the DNA to transcription factors and facilitates, or suppresses gene expression [106,107]. A well-characterized epigenetic modification is the acetylation of lysine residues within the N-terminal tail of core histone proteins [106]. The acetylation and the deacetylation of histones has to be precisely balanced to maintain normal physiological and developmental processes [108]. Disruption of this equilibrium is associated with anomalies in development, health, behavior, and other life-history traits [109–111]. For example, a disturbed ratio of acetylation and deacetylation of histones in the fruit fly *Drosophila melanogaster* affects survival and development, while in the flesh fly *Sarcophaga bullata*, such dysregulations interfere with the pupal diapause [112–115]. In honey bees (*Apis mellifera*), a reduced deacetylation induces neurological defects, but at the same time can

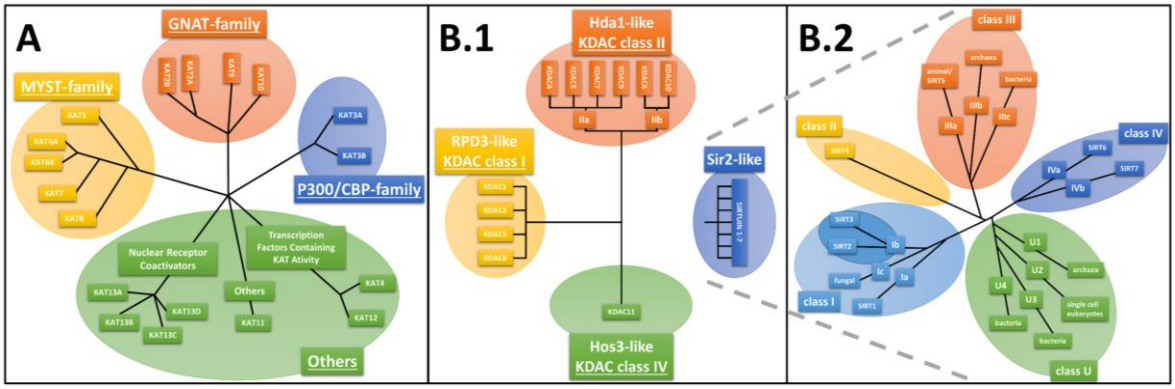

Figure 2. Classification of (A) Type A lysine acetyltransferases [176], (B.1) histone deacetylases with sirtuins as an independent group of deacetylases [168] and (B.2) sirtuins [177,178].

promote queen bee development [116,117]. The importance of this epigenetic mechanism in aphids has been discussed under different perspectives, including wing and reproductive polyphenism [118–120]. However, the particular significance of histone acetylation in normal aphid development remains unresolved.

The reversible acetylation of histone residues is catalyzed by enzymes originally called histone acetyltransferases and histone deacetylases [106]. Today, these enzymes are also known as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), because they additionally target an abundant number of non-histone proteins [121–125]. A remarkably diverse spectrum of highly conserved KATs and KDACs has been identified in many organisms (Fig. 2) [121,126–128]. Most KATs belong to one of three protein families: the GCN5-related N-acetyltransferases (GNAT) family (KAT1-2); the p300/CREB-binding protein (p300/CBP) family (KAT3A, KAT3B); and the MOZ/Ybf2/Sas2/Tip60 (MYST) family (KAT4-8) (Fig. 2A) [121,127,129]. The 11 known KDACs can be subdivided into three families: Class I or Rpd3-like proteins, comprising Rpd3/KDAC1, KDAC2, KDAC3 and KDAC8; Class II or Hda1-like proteins, comprising KDAC4-7; and Class IV or Hos3-like proteins with KDAC11 as its only resident (Fig. 2B) [121]. The sirtuins (Sir1-7), the fourth subgroup of deacetylation enzymes, was previously referred to as Class III KDACs, but they appeared to be mechanistically and structurally distinct from the other KDACs. Consequently, they are now considered an independent group of proteins (Fig. 2C) [121,130–132].

2.1.1 *KATs and KDACs in A. pisum - detection and characterization*

In the present work, the NCBI database and the genome of *A. pisum* have been screened for KAT and KDAC (incl. sirtuin) genes and motifs to identify relevant gene and protein sequences [14,133]. This bioinformatics approach revealed 18 KAT-related and 16 KDAC-related sequences within the pea aphid genome. These results support previous studies suggesting an extended repertoire of chromatin-remodeling proteins in the pea aphid [14,103,133]. Assembly and annotation of whole genomes, including the pea aphid genome, are performed automatically and remain a huge computational challenge, which can be biased

in various ways [134]. Coverage bias or two paralogous genes collapsed into a single gene by automatic assembly decrease the number of predicted genes within a draft genome. Conversely, splitting of allelic variants into separate paralogous loci (split genes) or the cleavage of gene sequences and the subsequent association of the resulting fragments onto more than one scaffold or contig (cleaved genes) leads to substantial overestimation of gene diversity [135]. With this in mind, the observed sequences have been further characterized – by manual examination of the underlying genomic information, protein domain analysis, and phylogenetic comparisons. Eventually, they were examined by *in vitro* detection via PCR and Sanger-sequencing [103,136]. In this detailed analysis, Kirfel et al. (2019) for the first time revealed evidence for split genes (e.g. *hdac8* and *rpd3*) and cleaved genes (e.g. *kat6b* and *kat7*) in the pea aphid genome assembly [103]. Nevertheless, several KATs and KDACs were clearly identified by the phylogenetic analysis and proven experimentally. One *hat1* sequence and three *kat2b* sequences (GNAT family) have been cloned and sequenced. A KAT2A protein sequence was found and could not be ruled out by the bioinformatics analysis, yet an *in vitro* validation of its mRNA failed. From the MYST family, one sequence each representing *kat5, kat6b* and *kat7* was confirmed *in silico* as well as *in vitro* [103]. One member of the p300/CBP family was recognized and analyzed in detail by Kirfel et al. (2020) [136]. As mentioned previously, some KDAC sequences found are most likely split gene events, e.g. only one transcript each out of two predicted *hdac8* and four predicted *rpd3* sequences (both Class I KDACs) could be verified experimentally. Conversely, a true gene duplication event was observed in the case of the two KDAC6-like sequences (Class II KDACs), which were found both bioinformatically and experimentally. Moreover, there is strong evidence for the existence of *kdac3* (Class I), *kdac4* (Class II) and *kdac11* (Class IV), but not for homologs of KDAC2, KDAC5, KDAC7 and KDAC9. Four sirtuins were cloned and sequenced following the bioinformatic identification (*Sir1, Sir4, Sir6* and *Sir7*). The mitochondrial *Sir5* was found *in silico* but its mRNA transcript could not be confirmed in the lab [103]. It was demonstrated in Kirfel et al. (2019) that previous studies possibly overestimated the diversity of histone-modifying enzymes in some respect, but also showed that the repertoire of KATs and KDACs, indeed, has been extended in some cases (e.g. *kdac6* and *kat2b*) in *A. pisum* [103,127].

2.1.2 INHIBITING HISTONE MODIFICATION ENZYMES SIGNIFICANTLY EFFECTS APHID FITNESS

In this thesis, the role of histone modification in normal aphid development was investigated, in order to estimate the importance of this epigenetic modification for polyphenism, as suggested in previous studies [118–120]. Epigenetic modifications, and protein acetylation, in particular, are key regulatory adaptations in many biological processes [122,123,137]. Inhibition of this mechanism could lead to a dramatic loss of fitness [113]. Notably, this result may become the key to approaching a novel target system for insecticidal agents and strategies, apart from the most important classical targets like the nervous system

or chitin biosynthesis. Therefore, aphids were fed on an artificial diet containing either epigenetic multiple ligand (epi-ML) or suberoylanilide hydroxamic acid (SAHA), two chemicals inhibiting the activity of KATs or KDACs, respectively. Upon inhibitor ingestion, survival was reduced by up to 70% (SAHA) and 50% (epi-ML), respectively. Furthermore, a reproductive delay of up to 2 days was observed in a concentration-dependent manner [103]. Likewise, the inhibition of KDACs reduced survival and caused developmental arrest in *D. melanogaster*, the frog *Xenopus laevis* or the starfish *Asterina pectinifera* [113,138–140]. Despite the reduced development of *A. pisum* nymphs following SAHA treatment for KDAC inhibition, such aphids gained a greater body weight than their untreated peers [103]. This might result from nonspecific cell growth comparable to that reported for *X. laevis* [137]. Contrary to what has been observed for SAHA, inhibition of the KAT enzymes by epi-ML led to significantly smaller body size and lower body weight of the aphids [103]. These strong effects on survival, reproduction, growth, and development, as described in Kirfel et al. (2019), experimentally proved the importance of histone modification in several life-history traits in aphids. However, a polyphenetic response resulting from exposure to these two inhibitory chemicals was not discovered [103]. It is possible that this phenomenon is governed by other epigenetic modifications. Such an effect was shown by results of Dombrovsky et al. (2009) [142], who demonstrated that inhibition of DNA methylation using RG108 (*N*-phthalyl-L-tryptophan) or zebularine (pyrimidin-2-one-*β*-D-ribofuranoside) promoted wing development in *A. pisum*.

The chemical disturbance of the entire histone acetylation system by SAHA and epi-ML, respectively, offers the opportunity to evaluate a possible role of this epigenetic modification in the aphid's biology in general. Notably, this system also bears potential as a novel target for aphid control. To go more into detail, RNA interference was used to determine the function and importance of the previously identified genes involved in histone modification (see 2.3.1). RNAi is a preserved biological response mechanism to exogenous double-stranded RNA. It is a mechanism for sequence-specific gene silencing present in most eukaryotic organisms. RNAi controls gene expression and facilitates resistance to endogenous parasitic as well as exogenous pathogenic nucleic acids [143–147]. Silencing of the KATs *hat1*, *kat2b1*, *kat2b2*, *kat8* and the KDACs *kdac3*, *kdac6*, *kdac8* and *sir1* did not induce significant changes to the monitored parameters like survival, development or offspring production. Interestingly, silencing the KDAC *Rpd3* led to a small but significant number of prematurely born, nonviable offspring [103]. Considering that embryos of *rpd3*-deficient *D. melanogaster* develop segmentation defects and also failed to hatch, these results suggest that Rpd3 is essential in aphid embryogenesis and eclosion [103,148]. Interference with the histone acetyltransferase *kat6b* significantly extended the aphid lifespan. Inhibition of *kat7* mRNA resulted in the production of considerably more offspring compared to the controls. This is in line with studies in yeast, *D. melanogaster*, *Caenorhabditis elegans,* and mice, reporting a prolonged survival

after the loss of histone acetylation [149,150]. A lack of histone acetylation, either through impaired acetyltransferases or by an accelerated deacetylation process, is suggested to stabilize gene expression and thus, like in aphids, increase longevity [151–154]. The mechanism by which KAT7 promotes offspring production in aphids remains unclear. However, such beneficial effects of the inhibition of histone acetylation may be limited to certain acetyltransferases since the attenuation of the acetyltransferase p300/CBP appeared to be detrimental to the aphids' fitness [136].

2.1.3 About p300/CBP in aphids – a universal transcriptional co-regulator

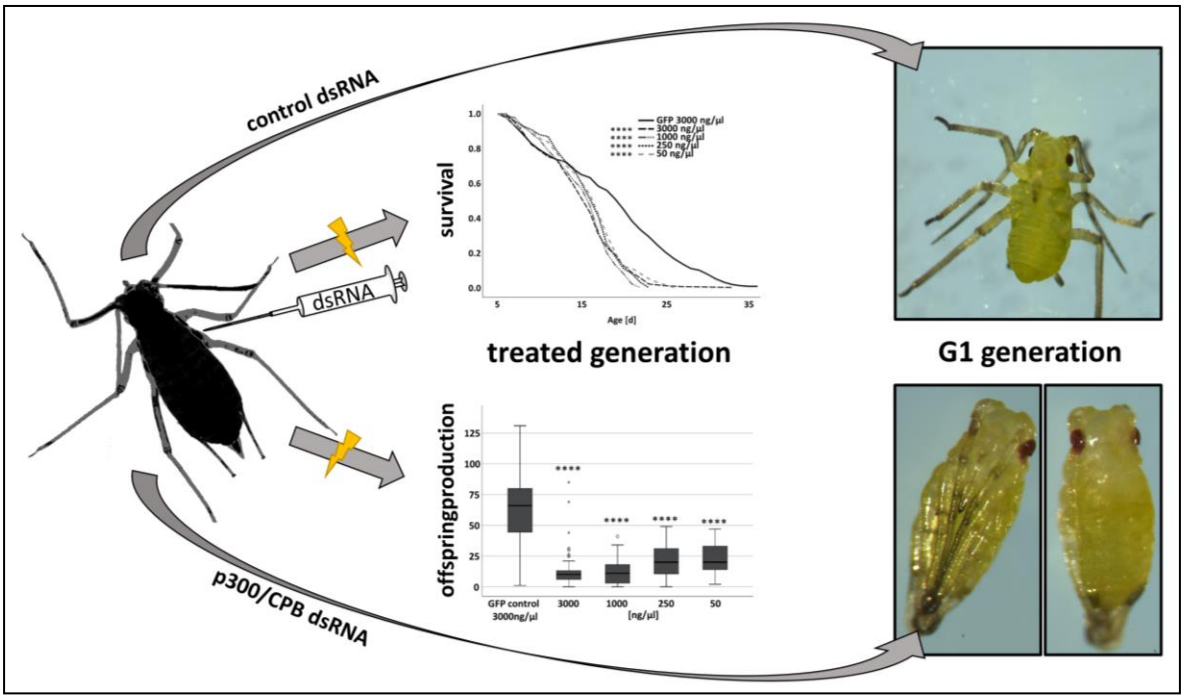

Figure 3. Scheme of the experimental procedure used for dsRNA injection. Injection occurred between the meso- and metathorax of a 5-day-old mother. The effect of different p300/CBP dsRNA concentrations on survival and the production of offspring is displayed in graphs. Additionally, regular offspring compared to premature offspring born after the injection of control dsRNA (top-right) and p300/CBP dsRNA (down-right), respectively, is shown.

Although historically associated with histone acetylation, p300/CBP is now known to be important for numerous acetylation processes and signaling pathways [155–158]. With more than 400 protein targets resulting in the acetylation of over 100 protein substrates, this enzyme has been shown to be essential in many ways for growth and development in multicellular organisms [156,159–162]. In humans, p300/CBP has been demonstrated to play important roles in various forms of cancer [163]. In *D. melanogaster*, the German cockroach *Blattella germanica* and the red flour beetle *Tribolium castaneum*, the loss of p300/CBP activity has been shown to cause severe embryonic or postembryonic deformation [115,164–167]. Similar to the other KATs, nothing was known about the function of p300/CBP in aphids. The inhibition of most KATs in aphids through dsRNA injection exhibit no or rather mild incisions in the observed life-history traits [103]. Conversely, the injection of p300/CBP dsRNA had manifold and fatal consequences in *A. pisum* (Fig.3) as demonstrated in Kirfel et al. (2020) [136]. This result

strongly supports the conserved function of p300/CBP in fundamental regulatory and cellular processes as reported for other organisms [164,166]. E.g. in *D. melanogaster*, *C. elegans* and mice, p300/CBP seems to be essential during embryogenesis. If dysregulated, it causes lethality and severe developmental defects in these organisms [165,167,168]. Kirfel et al. (2020) revealed that treated aphids produced less offspring, gave birth to an astonishing number of premature nymphs, and showed that the attenuation of p300/CBP triggers the retention of embryos by their mothers (Fig. 3) [136]. Such an impact can be explained by a disrupted embryogenesis. It can also be caused by reduced tissue integrity within the aphids' ovaries observed in this study upon p300/CBP manipulation. Along with additional effects perceived, such as the dramatic truncation of the reproductive phase and the dark-green hyperpigmentation of the aphid body, it was concluded in Kirfel et al. (2020), that sufficient evidence is provided for the induction of senescence by p300/CBP mitigation [136,169]. This is in agreement with other studies associating p300/CBP with an accelerated biological aging process [136,169]. Essential roles of p300/CBP in the senescence of human cells, the apoptosis of insect cells, and the disabling of lifespan extension in *C. elegans* have been reported, as well as a correlation of age and gene expression levels of p300/CBP in mice [170–174]. The hyperpigmentation of the aphids' bodies, which can also be observed in aging aphids under normal conditions, is suggested to result from encapsulation and melanization of particles. This is suggested to be one of the few active immune response mechanisms in aphids [175-178]. From experiments in *T. castaneum* it is known that the knockdown of p300/CBP affects the expression of more than 1,300 genes, which trigger an enhanced melanization in the midgut of the beetles as a consequence of changes in innate immunity, pigmentation, and metabolism [166]. Hence, future investigations should additionally consider a correlation between the dysregulation of p300/CBP and aphid immunity. Apart from this, depletion of p300/CBP altered both foraging behavior and food intake in *Camponotus floridanus* and *B. germanica*. Moreover, modulation of gluconeogenesis and lipidogenesis was observed in the latter species, whereas the stability of nutritional storage proteins was affected in *Bombyx mori* [164,179,180]. Malnutrition may account for or, at least, exacerbate the detected developmental aberrations and the lifespan reduction. However, such an explanation seems to be less probable for aphids, because no obvious changes in feeding behavior, body weight, and body size were observed in this study.

2.1.4 dsRNAs as bioinsecticides – RNAi as an alternative aphid management strategy

RNAi is regularly used to protect beneficial insects from pathogens or parasites like the Israeli acute paralysis virus, the Varroa mite (*Varroa destructor*), or the microsporidian parasite *Nosema ceranae* [181–185]. It was further considered as an alternative, environmentally friendly approach to control (pest) insects themselves [186]. Indeed, Baum et al. (2007) [187] and Mao et al. (2007) [188] demonstrated that insect-derived dsRNA expressed *in planta*

exhibit a significant degree of protection against the western corn rootworm (WCR) *Diabrotica virgifera* and the cotton bollworm *Helicoverpa armigera*, respectively. The only commercially available product using RNAi as the active mechanism against insects is SmartStax PRO maize that is expressing *DvSnf7* dsRNA, which was estimated to reduce WCR appearance by up to 95% [189]. Although feasible for many insects [144], some Lepidopteran and Hemipteran species, including aphids, appear to be recalcitrant in their response to environmental RNAi [101,147,190,191]. Previous studies aiming to establish RNAi-mediated aphid control focused on target systems that are well known in the context of pest control. Such studies were looking for genes related to insect digestion, respiration, and chitin biosynthesis. However, they were of debatable success [192–197]. Various reasons for a reduced susceptibility or resistance to RNAi were suggested, including natural barriers, such as the insect gut pH value, dsRNA degradation by nucleases, the accumulation of dsRNA in endosomes, and SID-1 deficiency. In other words, those mechanisms are affected that disturb normal uptake and (systemic) transport of dsRNA [102,198,199]. Additionally, RNAi-mediated gene silencing could be counteracted by regulatory feedback mechanisms, which are able to compensate for the function of the silenced gene. In this context, the gene's transcription rate can be increased, or the up-regulation of other genes can be expedited. Alternative explanations for a less effective RNAi scenario comprise low expression or malfunction of RNAi enzymes or compounds [180].

The first obstacle for efficient RNAi, which has eventually been identified in aphids, was an extracellular dsRNase in the gut. It significantly and non-specifically degrades ingested dsRNA molecules [102,200]. Further, although all compounds of the siRNA pathway and related transport proteins are present in aphids, it remains controversial how and if these genes are regulated upon dsRNA delivery [102,201,202]. Remarkably, the examination of enzymes of the second RNAi pathway, the miRNA pathway, which can cause translational instead of transcriptional repression, has experienced an extensive expansion in *A. pisum*. The exposure of aphids to different kinds of dsRNA was followed by a vast, but inconclusive expression crescendo of several components of this pathway [201,203]. In this thesis, only a partial knockdown (~30%) of the expression levels of the *p300/CBP* mRNA was detected, which was

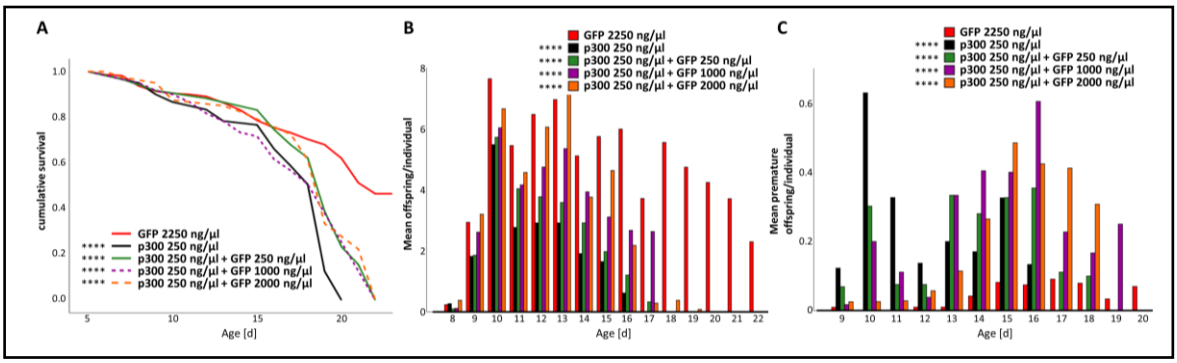

Figure 4. Effects of RNAi after injection of p300/CBP dsRNA and GFP control dsRNA mixtures in different ratios (pure, 1:1, 1:4, 1:8). (A) survival, (B) mean number offspring/day, (C) mean number premature offspring/day.

strictly in line with results found in many other studies on the pea aphid. Considering the extension of the miRNA pathway, it is worth suggesting that effects observed upon dsRNA injection in the pea aphid, at least to some degree, is based on a translational instead of transcriptional repression leading to only minor mRNA reductions hardly detectable by quantitative PCR [136]. Even if it was clearly demonstrated that there were only minor changes in the expression levels of the *p300/CBP* mRNA, the consequences on various *A. pisum* life-history traits were significant. However, a strong phenotypic effect even if, or especially if, the knockdown is only partial, is a prerequisite for a promising candidate gene in terms of RNAi-based insect pest control [200]. As pointed out, all enzymes of the RNAi pathways are present, but the overall expression levels of relevant enzymes as well as the fate of dsRNAs in *A. pisum* and other aphids largely remains unknown. Kirfel et al. (2020) demonstrated that even very low amounts of dsRNA (1.25 ng) can have tremendous effects on the life-history traits of aphids [136]. Surprisingly, the injection of larger amounts of dsRNA (75 ng) did not result in stronger effects in terms of mortality, offspring count, and viability of emerging nymphs [136]. These results may be an indication for low expression levels of participating enzymes and consequentially rapid saturation of the RNAi machinery. This hypothesis is supported by further experiments deploying mixtures of GFP and *p300/CBP* dsRNA in different proportions (1:1, 4:1 and 8:1, respectively). A rapid competitive inhibition of the RNAi pathways was observed (Fig. 4). Although it remains unclear, which part of the RNAi pathways (uptake, transport, core enzymes, etc.) is affected most seriously, this finding provides an alternative explanation for the variable efficacies of RNAi observed in aphids. At the same time, the assumption was corroborated that very strong phenotypic effects, necessary for RNAi-based aphid control, can be achieved by careful selection of a suitable target gene. It should not be neglected that the hypothesized fast and easy competitive inhibition of the RNAi machinery may come along with new challenges, especially for previously suggested delivery systems. For instance, modified viruses, which are commonly employed as transfer vectors, may not be a suitable tool for the control of aphids [98].

Table 3. Identity of the p300/CBP dsRNA construct to the mRNA of other aphid species

Aphid species	**Identity**	**Contig. nt (>21-mers) of *A. pisum* dsRNA similar to p300 mRNA of other species**
Cotton aphid (*Aphis gossypii*)	93%	38nt, 32nt, 29nt, 41nt, 33nt
Russian wheat aphid (*Diuraphis noxia*)	96%	44nt, 123nt, 43nt, 47nt
Green peach aphid (*Myzus persicae*)	97%	28nt, 44nt, 80nt, 77nt, 47nt, 27nt
Sugarcane aphid (*Melanaphis sacchari*)	93%	29nt, 38nt, 26nt, 29nt, 26nt, 38nt, 30nt
Corn leaf aphid (*Rhopalosiphum maidis*)	92%	29nt, 38nt, 92nt, 23nt, 33nt
Yellow sugarcane aphid (*Sipha flava*)	88%	26nt, 47nt, 22nt

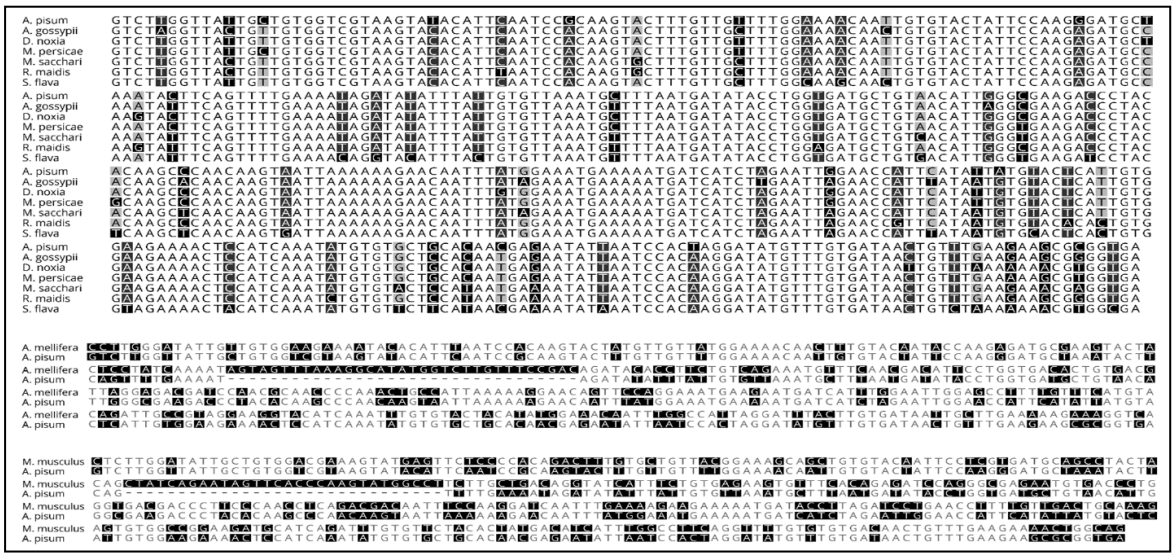

Figure 5. Alignments of the *A. pisum* dsRNA construct with sequence of the p300/CBP mRNA sequence of other aphids (*Aphis gossypii*, *Diuraphis noxia*, *Myzus persicae*, *Melanaphis sacchari*, *Rhopalosiphum maidis*, *Sipha flava*), the honey bee (*Apis mellifera*) and the house mouse (*Mus musculus*).

As discussed before, resistance is a major concern of every pest control product. To prevent resistance against dsRNA constructs, the use of long dsRNA has been suggested. Certainly, the longer the delivered dsRNA constructs, the less are the odds for mutations triggering resistance, but the higher the risk to target non-target genes in aphids or genes in non-target organisms [204]. The p300/CBP dsRNA construct used herein was >300 nucleotides long, and there is a high degree of identity to other aphid p300/CBP mRNA sequences (Tab. 3, Fig. 5). That means that the construct used herein is most likely not species-specific but presumably limited to the aphid family. In fact, no RNAi-relevant overlaps were identified to the mRNA of the beneficial insect *A. mellifera* and the mammal *M. musculus*. Consequently, those organisms are not targeted by the present dsRNA. Considering the strong phenotypic effects and the high specificity, the dsRNA molecules designed in this study are ideal candidates for use as alternative and aphid-specific crop protection products (Patent application: Methods of Multi-Species insect pest control #EP19209940).

2.2 FRIEND OR FOE? – THE APHID'S BACTERIAL PASSENGER *SERRATIA SYMBIOTICA*

A pest control strategy advocated for many years is the application of insecticides at the maximum permitted dose [205]. This practice is debatable, and a simple way to reduce insecticides is to apply the lowest possible, but still sufficient amount of insecticidal compound in the field [205,206,207]. To determine the appropriate amount of an insecticide to be applied, fundamental knowledge of factors influencing the susceptibility of the pest is mandatory. Contemporary approaches for IPM include the accurate identification of the pest to be controlled as well as an in-depth understanding of its biology and ecology [208]. For example, the presence of some bacteria in insects is associated with increased host susceptibility to insecticides due to overall reduced fitness of the host-insect [209–211]. In an opposite way, it

has been demonstrated that the bacterial symbionts of other insects such as the bean bug *Riptortus pedestris* [212], the oriental fruit fly *Bactrocera dorsalis* [213], or the mountain pine beetle *Dendroctonus ponderosae* [214], act as detoxifiers. Presumably, such symbionts originally might have enabled the host to overcome plant defenses, but eventually, they specialized in mediating insecticide resistance [215–220]. Consequently, the presence or absence of a highly-specialized symbiont in a specific pest may substantially alter the necessary amounts of insecticidal compounds to be applied in the field.

Outstanding examples of detoxifying bacteria are found in the genus *Serratia* [214,221-225]. One member of this genus, *S. symbiotica*, is one of the most frequent facultative symbionts in aphids, including the pea aphid [226]. *S. symbiotica* can protect aphids from parasitoids, predators, and heat stress [227–230]. Recently, it has been suggested to support the feeding on host plants by the excretion of digestive enzymes or through the suppression of plant defenses [231]. Therefore, the hypothesis investigated in this thesis was that *S. symbiotica* could also assist the detoxification of insecticides in *A. pisum*. Consequently, the insecticide resistance of aphid strains harboring this symbiont should be more pronounced. The influence of the aphids' symbiont on the susceptibility to common insecticides was assessed with respect to the overall impact of *S. symbiotica* on *A. pisum* fitness [233]. Therefore, aphids from a *Serratia*-positive stock population were sterilized. Using ampicillin, a *Serratia*-free line of *A. pisum* was established. Then, several fitness parameters (development, reproduction, size, longevity) of the newly established *Serratia*-free population were analyzed and compared to those of the original *Serratia*-positive population. Furthermore, a bioassay was established to test the efficiency of different insecticides covering a spectrum of insecticide classes, frequently used for aphid control [64,232,233]. Aphids were exposed to a neonicotinoid (imidacloprid: agonist on the nicotinic acetylcholine receptor), an organophosphate (chlorpyrifos-methyl: non-competitive inhibitor of acetylcholine esterase), a carbamate (methomyl: non-competitive inhibitor of acetylcholine esterase), a diamide (cyantraniliprole: agonist on the ryanodine receptor) and a tetramic acid derivate (spirotetramat: inhibitor of acetyl-CoA carboxylase). Three concentrations of each insecticide were evaluated in order to determine LC_{50} values for both aphid lines [233]. Contrary to the hypothesis, *S. symbiotica* infection in *A. pisum* led to severe fitness costs regardless of the insecticide treatment. Without treatment, aphids hosting the bacterial symbiont underwent a significantly prolonged development, they produced fewer offspring and were considerably smaller (lighter) than individuals from the sterilized, *Serratia*-free population. Following insecticide treatment, the mortality in the *Serratia*-positive aphid line was substantially higher than that one in the sterilized line. This result was confirmed for all insecticides tested, except for cyantraniliprole, especially when low concentrations of the substances were applied [233]. This is in line with results discovered in *Bemisia tabaci* or *Plutella xylostella*, where the existence

of a bacterial symbiont increases the susceptibility to chemical insecticides [210,234]. As previously mentioned, whether symbiotic bacteria are only beneficial or come with costs to their insect hosts depends on a complex interplay of diverse factors. The effect of a symbiont can change, depending on environmental conditions, the presence or absence of stressors [235,236]; and they can vastly differ depending on the host as well as the symbiont species [32,169,237] or even strains [238,239]. In several studies, a trade-off between the insect's endurance, offspring production, or development and their need to control their symbiotic community to ensure the success of both partners, has been demonstrated [169,237,240]. Likewise, it can be assumed that the fitness costs associated with the maintenance of *S. symbiotica* in aphids take part in the alteration of their ability to cope with chemical insecticides. Consequently, this study suggests that host sensitivity to insecticides is influenced by the introduction of a bacterial symbiont. In other words, the presence or absence of a particular symbiont may be a key factor for the efficacy of insecticide applications against insects in the field. This decisive result obtained in this thesis needs to be considered in future pest management strategies.

2.3 AMPs – ANTIMICROBIAL PEPTIDES EVOLVING INTO BIOINSECTICIDES

Antimicrobial peptides (AMPs) are short polypeptides with either a linear structure encompassing amphipathic *α*-helices, or a cyclic structure including *β*-sheets stabilized by disulfide bridges [241]. These peptides have been found in all living organisms ranging from bacteria to plants as well as vertebrates and invertebrates. As part of the organisms' innate immune system they exhibit antimicrobial activity, but additionally show antiviral or anti-inflammatory functions, etc. [242,243]. Depending on the individual biological surrounding, AMPs may act through numerous mechanisms including the formation of ion channels in the cell membrane; they may alter enzyme activity, inhibit protein folding and bind intracellular targets [244]. Although AMPs are under discussion as a replacement for classical antibiotics, only a few have officially been approved as such. This is mainly due to concerns about toxicity, cleavage, and stability [245–247]. To broaden the potential industrial use of AMPs, one attempt of this thesis was to evaluate the insecticidal activity of AMPs and assess their utility as bio-insecticides against aphids.

Insects rely exclusively on their innate immune system to fight infections with pathogens or parasites [248]. AMPs play central roles in the insects' immune response against intruding microbes [249–251]. Surprisingly, in the *A. pisum* genome, some genes for the detection and the elimination of microorganisms are absent, and only a weak immune response was detected following infection or stress treatments [249]. More precisely, the pea aphid genome lacks genes encoding typical antibacterial AMPs such as defensins. It is hypothesized that the reduction of the innate immune system co-evolved with the introduction of obligatory

endosymbionts in aphids [176,249,252,253]. These bacterial symbionts are responsible for the aphids' ability to survive on nutritionally poor diets [254,255]. Therefore, eradication of the endosymbiont by antibiotics leads to a dramatic loss of fitness and fertility [256,257]. Consequently, new strategies for aphid pest control could be developed, which selectively target the symbionts themselves or the bacteriocytes harboring these organisms.

To efficiently kill their prey, the venom of scorpions contains neurotoxins and other bioactive molecules, as well as a complex mixture of AMPs, optimized over millions of years in evolution [258]. The use of scorpion AMPs as antibiotics is still a challenge due to their mildly hemolytic activity, but they may also be an untapped source for novel peptides with insecticidal activity. In this study, their potential use as insecticides was investigated by testing their activity against the pea aphid and its bacterial symbiont, *in vitro* as well as *in vivo*. The AMPs used herein were part of the largely uncharacterized group of non-disulfide bridged peptides (NDBPs) [259] and have been isolated from the venom gland transcriptome of the Australian native scorpions *Urodacus yaschenkoi* and *Urodacus manicatus*. Naturally occurring scorpion AMPs and modified analogs thereof were fed to the pea aphid [260–265]. Survival and reproduction of the treated insects were analyzed, and the results were compared to treatments with three insect-derived AMPs (apidaecin, cecropin A, stomoxyn). Rifampicin, which inhibits the bacterial DNA-dependent RNA polymerase, was used as a control antibiotic, whereas the neonicotinoid imidacloprid was used as a control insecticide. Additionally, the impact of the AMPs on bacterial load of *B. aphidicola* and *S. symbiotica* have been investigated *in vivo* with quantitative PCR. *In vitro*, minimal inhibitory concentrations (MICs) against *S. symbiotica* were estimated using the cultivable strain CWBI-2.3*,* which is closely related to the *S. symbiotica* strain found in *A. pisum* [266]. The scorpion AMPs affected the survival of *A. pisum* to a varying extent, whereas none of the used insect AMPs exhibited lethal effects [261]. The observed modes of action of insect and scorpion AMPs are comparable, in general. However, while acting against a wide range of bacterial pathogens, insect AMPs do not seem to target eukaryotic cells [267–271]. In contrast, scorpion AMPs are devoid of this kind of specificity and have been shown to target bacteria, but also eukaryotic cells such as erythrocytes [258,268,270]. It has been suggested, that venom gland-associated AMPs not only protect the telson of scorpions against bacterial intruders. Additionally, they may influence the toxicity of the venom by destroying cell membranes and deploying entrance sites for the neurotoxins [268]. The observed decrease in aphid survival after AMP treatment could reflect the direct damage caused by scorpion AMPs to different cell-types, including gut cells and the bacteriocytes. This idea is supported by the fact that the AMPs influenced aphid viability in the feeding assay, although they were not active against *S. symbiotica* or *B. aphidicola in vivo*. Moreover, they did not exhibit activity against the cultivable *S. symbiotica* strain CWBI-2.3 or *Escherichia coli* (as a close relative to *B. aphidicola*) *in vitro* [260,261]. Nevertheless, most of

the scorpion AMPs, but not the insect AMPs, significantly reduced the concentration of the obligatory as well as facultative symbionts in the aphids. Still, the ability to affect eukaryotic cells may help the scorpion AMPs to penetrate the protective membranes and barriers provided by the aphids' bacteriocytes, thereby promoting the perceived antimicrobial activity of the scorpion AMPs in aphids. Notably, the aphids' bacterial symbionts were either immune against the tested insect AMPs or well protected by their bacteriocytes [261].

In summary, scorpion AMPs are found to be promising as potentially novel insecticidal compounds. They reduced the survival as well as the reproduction of the aphids after oral uptake, presumably through direct damage of the aphids' cells. Some of the AMPs examined, additionally decrease the amount of bacterial symbionts *B. aphidicola* and *S. symbiotica*. This also shows that the scorpion AMPs are functional even following exposure to the aphids' digestive system. Genetically modified plants, ectopically expressing the antifungal peptide gallerimycin, have been shown to be protected against fungal infection [272]. Likewise, this study showed the potential of scorpion AMPs acting as insecticidal compounds, which could be provided through insect pest-resistant GM crops, topical application, or even other methods previously discussed (see 1.2.2). Hence, future research has to examine suitable application techniques, ecotoxicology and persistence, side effects on the adapted crop or the environment as well as the degree of protection offered by the AMPs applied.

3. CONCLUSIONS AND FURTHER PERSPECTIVES

"when pests and diseases are causing major losses of biodiversity, and when we are increasingly aware of the contribution that plants make to our quality of life,[…], we simply have to be more cautious, more vigilant and more demanding, in tackling this major environmental risk." (Charles P. A. G., Prince of Wales at a Plant Health and Biosecurity Conference at the Royal Botanic Garden, Kew)

In recent years, the implementation of novel and secure insect management methods, or their improvement, became obligate for the entire agroindustry. This thesis focused on the development of alternative strategies for sustainable aphid management, which may stimulate the search for novel targets or pest control substances or may help to improve existing methods. The major subject of this thesis was the identification of the broad diversity and function of histone acetylation and deacetylation enzymes. It was postulated that this tightly controlled epigenetic machinery provides a promising target system for the development of novel insecticidal compounds. Surprisingly, this work exhibits the resilience of the epigenetic machinery against external manipulation, but also its weaknesses in terms of the highly connected gene node p300/CBP. The attenuation of p300/CBP has tremendous effects on life-history traits of *A. pisum*, corroborating the fundamental role of p300/CBP as a universal transcriptional co-regulator in insects. The RNAi-based insect pest control through transgenic plants may not be suitable for aphids for the reasons explained in previous sections (see 1.2.2), thus the utilization of dsRNA spray formulations with p300/CBP dsRNA as an active substance could provide a highly specific and environmentally sustainable method for aphid control. This work not only provides evidence for the potential of an RNAi-based pest management strategy but also revealed that this system may be prone to competitive inhibition and low expression levels of enzymes of the RNAi pathways. These new challenges need to be considered in future gene silencing experiments, the search for relevant target genes, the development of dsRNA delivery systems and the integration of this method in aphid management systems in general. As a second aspect, this thesis investigated one neglected aspect of aphid management: their bacterial symbionts. The presence of the facultative symbiont *S. symbiotica* increases the susceptibility of *A. pisum* to chemical insecticides. This knowledge opens up a new door to the optimization of the use of synthetic insecticides based on the symbiotic community of a specific aphid population and its resistance to different artificial stress agents. Additionally, the primary bacterial symbiont *B. aphidicola* itself provides a novel target for aphid pest control. Feeding scorpion-derived AMPs to *A. pisum* significantly decreased the fitness of this insect. Notably, this study disclosed the potential of scorpion AMPs as bioinsecticides. However, ecotoxicology and persistence, side effects on the adapted crop or the environment as well as the degree of protection offered by the applied AMPs have to be further examined.

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5. PUBLICATIONS

5.1 1ST PUBLICATION

1

Inhibition of histone acetylation and deacetylation enzymes affects longevity, development, and fecundity in the pea aphid (*Acyrthosiphon pisum*)

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RESEARCH ARTICLE

Inhibition of histone acetylation and deacetylation enzymes affects longevity, development, and fecundity in the pea aphid (*Acyrthosiphon pisum*)

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Abstract

Histone acetylation is an evolutionarily conserved epigenetic mechanism of eukaryotic gene regulation which is tightly controlled by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In insects, life-history traits such as longevity and fecundity are severely affected by the suppression of HAT/HDAC activity, which can be achieved by RNA-mediated gene silencing or the application of chemical inhibitors. We used both experimental approaches to investigate the effect of HAT/HDAC inhibition in the pea aphid (*Acyrthosiphon pisum*) a model insect often used to study complex life-history traits. The silencing of HAT genes (*kat6b*, *kat7*, and *kat14*) promoted survival or increased the number of offspring, whereas targeting *rpd3* (HDAC) reduced the number of viviparous offspring but increased the number of premature nymphs, suggesting a role in embryogenesis and eclosion. Specific chemical inhibitors of HATs/HDACs showed a remarkably severe impact on life-history traits, reducing survival, delaying development, and limiting the number of offspring. The selective inhibition of HATs and HDACs also had opposing effects on aphid body weight. The suppression of HAT/HDAC activity in aphids by RNA interference or chemical inhibition

revealed similarities and differences compared to the reported role of these enzymes in other insects. Our data suggest that gene expression in *A. pisum* is regulated by multiple HATs/HDACs, as indicated by the fitness costs triggered by inhibitors that suppress several of these enzymes simultaneously. Targeting multiple HATs or HDACs with combined effects on gene regulation could, therefore, be a promising approach to discover novel targets for the management of aphid pests.

KEYWORDS
acetyltransferase, deacetylase, epigenetics, life-history, RNA interference

1 | INTRODUCTION

In eukaryotic cells, gene expression can be regulated at the epigenetic level by chromatin modifications such as acetylation, methylation, phosphorylation, glycosylation, and ubiquitinylation (Yi, 2017). Many acetylation target sites have been found on proteins in all cellular compartments, and acetylation is, therefore, a major posttranslational modification involved in diverse biological processes (Ali, Conrad, Verdin, & Ott, 2018; Narita, Weinert, & Choudhary, 2019). Acetylation changes the charge of a protein, regulating its function by influencing its stability, enzymatic activity, subcellular localization, and interactions (Narita et al., 2019). One of the better-characterized epigenetic modifications is the acetylation of lysine residues within the N-terminal tail of the core histone proteins in the nucleosome (Bannister & Kouzarides, 2011; Burggren, 2017).

Histone acetylation is generally regulated by the opposing activities of two families of enzymes: histone acetyltransferases (HATs), also known as lysine acetyltransferases or KATs, and histone deacetylases (HDACs), also known as lysine deacetylases or KDACs (Ali et al., 2018; Bannister & Kouzarides, 2011). HATs catalyze the transfer of the acetyl group to the lysine side chain, and HDACs perform the reverse reaction (Bannister & Kouzarides, 2011). Lysine acetylation weakens the interactions between histones and DNA, thus facilitating gene expression by allowing transcription factors to gain access (Mukherjee, Fischer, & Vilcinskas, 2012; Patel, Pathak, & Mujtaba, 2011; Verdin & Ott, 2015). In contrast, the deacetylation of histones by HDACs makes DNA less accessible to transcription factors and this usually leads to the suppression of gene expression (Bannister & Kouzarides, 2011). A fine balance between HATs and HDACs maintains normal physiological and developmental processes (Haberland, Montgomery, & Olson, 2009) but any disruption of this balance can cause severe changes in behavior, development, health, and other life-history traits (Bassett, & Barnett, 2014; Damjanovski, Sachs, & Shi, 2000; Schneider et al., 2013). HATs and HDACs were the first enzymes shown to modify histones, but they can also target an abundant number of nonhistone proteins (e.g., cytoskeletal proteins; Drazic, Myklebust, Ree, & Arnesen, 2016; Narita et al., 2019; Ohguchi, Hideshima, & Anderson, 2018; Rahhal & Seto, 2019). Remarkably, some HDACs can even possess other enzymatic activities in addition to deacetylation (Bheda, Jing, Wolberger, & Lin, 2016; Cao et al., 2019; Narita et al., 2019). This shows the complexity between the nature of these enzymes, their multiple targets and biological processes they regulate. In this study, we broadly refer to Nε-lysine acetylation as a modification of histone proteins unless otherwise indicated.

Many studies have documented the diversity of HDACs and HATs (Crump et al., 2011; Verdin & Ott, 2015; Wang et al., 2009). The 11 known categories of HDACs are grouped into three subfamilies: Class I or Rpd3-like proteins (Rpd3/HDAC1, HDAC2, HDAC3 and HDAC8), Class II or Hda1-like proteins (HDAC4–7, HDAC9, and HDAC10), and Class IV or Hos3-like proteins (HDAC11) as shown in Figure S1 (Ali et al., 2018).

The sirtuins (Figure S2) are an independent group of seven categories of deacetylases formerly assigned as Class III HDACs (Ali et al., 2018; Frye, 2000; Greiss & Gartner, 2009; Nakagawa & Guarente, 2011). They suppress gene expression at telomeres and within recombinant DNA clusters among other genomic regions (Dang, 2014; Imai & Guarente, 2016). The naming convention for yeast and fruit fly (*Drosophila melanogaster*) sirtuins is the abbreviation Sir (silent information regulator) followed by a number, whereas SIRT is used in mammals (Szućko, 2016). Most HATs are assigned to one of three classes: the GCN5-related *N*-acetyltransferases (GNAT) family; the p300/CREB-binding protein (p300/CBP) family; and the MOZ/Ybf2/Sas2/Tip60 (MYST) family (Ali et al., 2018; Verdin & Ott, 2015).

The inhibition of HATs and HDACs at the messenger RNA (mRNA) or protein levels is a powerful approach for the analysis of epigenetic mechanisms (Glastad, Hunt, & Goodisman, 2019). Some insects are well-established models for studying epigenetic mechanisms including histone acetylation/deacetylation (e.g., *D. melanogaster*, *Tribolium castaneum*, and *Galleria melonella*; Bingsohn, Knorr, & Vilcinskas, 2016; Gegner et al., 2019; Heitmueller, Billion, Dobrindt, Vilcinskas, & Mukherjee, 2017; Mukherjee et al., 2012; Mukherjee, Twyman, & Vilcinskas, 2015; Vilcinskas, 2016). Histone modifications regulate genes controlling key life-history traits in many insects (Burggren, 2017). For example, histone acetylation/deacetylation affects survival and development in fruit flies and pupal diapause in the flesh fly *Sarcophaga bullata* (Kang, Marischuk, Castelvecchi, & Bashirullah, 2017; Pile, Lee, & Wassarman, 2001; Reynolds, Bautista-Jimenez, & Denlinger, 2016; Roy & Palli, 2018). Furthermore, the inhibition of HDACs promotes queen development in honey bees (*Apis mellifera*) but can also induce neurological defects (Lockett, Wilkes, Helliwell, & Maleszka, 2014; Spannhoff et al., 2011).

Here we investigated the effect of inhibiting HATs/HDACs in the pea aphid (*Acyrthosiphon pisum*), a widely used hemipteran model of symbiosis and insect–plant interactions with a wide range of available genomic resources (Rider, Srinivasan, & Hilgarth, 2010; Skaljac, 2016; Skaljac, Kirfel, Grotmann, & Vilcinskas, 2018; Skaljac, Vogel, Wielsch, Mihajlovic, & Vilcinskas, 2019; The International Aphid Genomics Consortium, 2010). The epigenetic basis of the environmentally-triggered wing and reproductive polyphenism in aphids has been reported (Baudach & Mukherjee, 2016; Gran, tham, Brisson, Tagu, & Le Trionnaire, 2015; Srinivasan & Brisson, 2012) but the role of HATs/HDACs has not been considered in detail. We, therefore, used RNA interference (RNAi) and chemical inhibitors to suppress the activity of these enzymes and investigated the effect on life history and fitness parameters such as longevity, development, and fecundity.

2 | MATERIALS AND METHODS

2.1 | Selection and evaluation of genes involved in histone acetylation/deacetylation

The *A. pisum* genome was screened for genes encoding three major groups of histone modification enzymes: acetyltransferases, deacetylases, and sirtuins (The International Aphid Genomics Consortium, 2010). We also took into account previously reported genes related to HDAC and HAT activity, such as *sap18* (HDAC complex subunit) and *kat14* (ATAC complex subunit; Rider et al., 2010; The International Aphid Genomics Consortium, 2010). In addition, human homologs of HDACs and HATs were screened against the *A. pisum* genome sequence using BLASTP (Altschul, Gish, Miller, Myers, & Lipman, 1990) to identify additional relevant genes.

Relevant protein domains were predicted using the Pfam database (El-Gebali et al., 2019) and cross-validated using the NCBI conserved domains database (Marchler-Bauer et al., 2017). Sequence similarities between HATs/HDACs and sirtuins in *A. pisum* and a wide range of other species were investigated by phylogenetic analysis. Multiple sequences were aligned using MUSCLE (Edgar, 2004) and phylogenetic trees were constructed using the RAxMl plug-in (Stamatakis, 2014) for Geneious v10.2.4 with default parameters, which allowed the trees to be annotated and displayed (https://www.geneious.com).

2.2 | RNA extraction, target gene identification, and double-strand RNA (dsRNA) synthesis

Total RNA from pools of 10 aphids was extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA (100 ng) was transcribed using the ReverdAid First Strand complementary DNA (cDNA) synthesis Kit (Thermo Fisher Scientific, Germany) and gene-specific primers were designed using Primer3 v4.1.0 (http://primer3.ut.ee/) to amplify specific regions of the resulting cDNAs. To verify the genes encoding *A. pisum* HDACs and HATs, the amplicons were cloned and sequenced as previously described (Skaljac et al., 2018). Primers and accession numbers for all HDAC and HAT sequences used in this study are listed in Table S1.

Appropriate PCR templates for gene silencing experiments were generated with gene-specific RNAi primers (designed using Primer3 v4.1.0 and purchased from Sigma-Aldrich, Germany), including in each case, a 5′ T7 promoter sequence. The dsRNA constructs were designed to be 300–500 bp in length with a GC-content of 40–60%, covering parts of the open reading frame. These constructs were checked for off-targets by screening against the entire pea aphid genome. The resulting amplicons for RNAi experiments were cloned and sequenced as previously described (Skaljac et al., 2018). The verified plasmid vectors were used as PCR templates to release amplicons for in vitro transcription using the primers are listed in Table S2. The amplicons were separated by gel electrophoresis, excised from the gel and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and then used for dsRNA synthesis with the Ambion MEGAscript T7 Kit (Applied Biosystems). The synthesized dsRNA was purified by isopropanol precipitation and washed with ethanol. The pellet was resuspended in 30–50 µl nuclease-free water and stored at −20°C.

2.3 | Chemical inhibitors of HDACs and HATs

We used the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), ≥98% purity from Cayman Chemicals, Estonia (CAS number 149647-78-9) and the HAT inhibitor epigenetic multiple ligands (epi-ML) from Merck, Germany (CAS number 1020399-52-3) as previously described (Mukherjee et al., 2012). For both inhibitors, a stock solution was prepared in 1% dimethyl sulfoxide (DMSO; Carl Roth, Germany) before the preparation of working dilutions.

2.4 | Aphid rearing, injection, and feeding assays

Parthenogenetic *A. pisum* clone LL01 was reared under constant conditions on the host plant *Vicia faba* var. *minor* as previously described (Luna-Ramirez, Skaljac, Grotmann, Kirfel, & Vilcinskas, 2017; Will, Schmidtberg, Skaljac, & Vilcinskas, 2017). Age-synchronized aphids were used in all experiments (Sapountzis et al., 2014). In the RNAi experiments, 5-day-old aphids were injected using glass capillaries held on an M3301 micromanipulator (World Precision Instruments). The aphids were injected laterally, between the middle and hind legs, with 25 nl of the dsRNA preparation (3 µg/µl) targeting the gene of interest or green fluorescent protein (GFP) as a negative control. Water injections were used as an additional control. We injected a total of 60 aphids per treatment, comprising three independent biological replicates of 20 aphids each. The injected aphids were reared individually until death in Petri dishes containing 1% agarose gel and *V. faba* leaves (Sapountzis et al., 2014; Will & Vilcinskas, 2015). Injection assays were used in RNAi experiments to avoid the degradation of dsRNA after exposure to aphid salivary secretions in feeding assays, as previously reported (Christiaens, Swevers, & Smagghe, 2014; Sapountzis et al., 2014; Singh, Singh, Mogilicherla, Shukla, & Palli, 2017). Aphid survival and offspring production were monitored daily to determine the effect of our experiments on development and reproduction (Skaljac et al., 2018). Developmental effects were determined by tracking the start of reproduction and the number of premature offspring, whereas the effect on reproduction was determined by tracking the total number of offspring and the number of offspring per day. Premature nymphs were not viable after eclosion and their antennae and legs

remained folded (Will et al., 2017). Newly emerged nymphs were counted daily and removed. Fresh Petri dishes containing *V. faba* leaves were provided regularly to ensure the aphids were maintained in an ideal environment.

The chemical inhibitors were orally delivered during feeding assays. *A. pisum* nymphs (48-hr old) were fed in modified chambers (Sadeghi, Van Damme, & Smagghe, 2009) for 5 days on a specialized AP3 diet (Table S3: Febvay, Delobel, & Rahbé, 1988) mixed with the inhibitors (10, 50, and 150 µg/ml for SAHA; 5, 10, and 25 µg/ml for epi-ML) or a control treatment (AP3 mixed with 0.5% DMSO). Ten nymphs were placed in each chamber and five replicates were tested per treatment. Each experiment was conducted with three biological replicates. The mortality of aphids was scored daily. Subsequently, 20 aphids that survived the 5-day feeding treatment were randomly selected and images were acquired under a Leica MZ 16 FA stereomicroscope to measure the body size (length and width). We also measured the body weight of the same individuals. Later, the aphids were transferred to agar plates as described above. These aphids were monitored daily for another 10 days to determine the effect of the inhibitors on survival and offspring production.

2.5 | Quantitative PCR (qPCR)

Aphids (whole bodies) previously injected with dsRNA were collected 48 hr postinjection into NucleoSpin RNA Kit lysis buffer (Macherey-Nagel). Insects previously fed with chemical inhibitors were dissected in 70% ethanol and their tissues (head, gut, embryos, and residual carcass) were immediately transferred to the lysis buffer. Three biological replicates of each sample containing pooled whole body or tissue samples from 10 aphids were used for total RNA extraction and cDNA synthesis as described above.

The expression of all target genes was evaluated by qPCR using the $\Delta\Delta C_t$ method (Pfaffl, 2001) and the StepOnePlus Real-Time PCR System (Applied Biosystems). The 10 µl reaction mixture comprised 2 µl of the cDNA template (diluted 1:4 with RNAse-free water before qPCR), 10 µM of each specific primer, and 5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems). Each reaction was heated to 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 59°C for 60 s. Melting curve analysis was performed by increasing the temperature from 59°C to 95°C for 15 s, cooling to 59°C for 60 s and heating again to 95°C in 0.3°C steps. The qPCR primers (Table S2) were designed using PrimerQuest (Integrated DNA Technologies; http://eu.idtdna.com/PrimerQuest) as previously described (Koressaar, & Remm, 2007; Untergasser et al., 2012).

The expression of all target genes was tested in duplicate for dissected tissues and in triplicate for whole-body extracts. Expression levels and statistically significant differences were calculated using the StepOnePlus Real-Time PCR System and REST2009 software (Pfaffl, Horgan, & Dempfle, 2002). Data were normalized using the reference genes *actin* (ACYPI000064) and *rpl32* (ACYPI000074) encoding ribosomal protein L32 (Sapountzis et al., 2014). In this study, the tissue-specific gene expression was monitored in the experiments with chemical inhibitors assuming that the activity of these compounds could have been restricted to specific tissue following the oral delivery (e.g., gut or head). On the other hand, dsRNAs injected in the hemolymph are expected to trigger a systemic RNAi effect that will eventually affect the whole body.

2.6 | Data analysis

Data were analyzed using IBM SPSS Statistics v25 (Armonk). The statistical significance threshold was $p < .05$ for most of the tests, except the two-way analysis of variance (ANOVA) where the threshold was $p < 0.001$. ANOVA was used to analyze the number of offspring per day. Survival was tested by nonparametric Kaplan–Meier survival analysis. The logrank test was used to determine differences in survival and the start of reproduction between groups. The number of offspring (premature and normal offspring), body weight, and size were analyzed using the Mann–Whitney *U* test for nonparametric data and Student's *t* test for normally distributed data.

3 | RESULTS

3.1 | Phylogenetic analysis and domain characterization of *A. pisum* HDACs and HATs

3.1.1 | HDACs

Screening the NCBI database and *A. pisum* genome revealed 11 pea aphid HDAC sequences. Seven were assigned to Class I/Rpd3-like (four representing Rdp3/HDAC1, one representing HDAC3, and two representing HDAC8), two were assigned to Class II/Hda1-like (one representing Class IIa/HDAC4 and two representing Class IIb/HDAC6), and one was assigned to Class IV/Hos3-like (HDAC11). The *A. pisum* genome does not appear to encode homologs of HDAC2, HDAC5, HDAC7, or HDAC9 (Figure 1; Table S1 and Figure S1). We experimentally confirmed the presence of a single transcript of *rpd3/hdac1*, *hdac3*, *hdac4*, *hdac8*, and *hdac11*, as well as two paralogous *hdac6*-like sequences (Figure 1a). The identified *A. pisum* HDAC sequences clustered phylogenetically together in the corresponding subgroups with sequences from other species, including the closely-related green peach aphid *Myzus persicae* and Russian wheat aphid *Diuraphis noxia* (Figure 1a).

Domain analysis revealed the presence of an arginase HDAC superfamily domain in each of the identified HDAC sequences, including the active-site residues and the zinc-binding sites (Figure 1b). The NCBI conserved domains search identified a specific HDAC Class I domain for one of the Rpd3 sequences (XP_016659248) which was not found in other Rpd3 sequences in the databases. In addition, specific HDAC3, HDAC Class IIa, and HDAC Class IV domains were found in HDAC3, HDAC4, and HDAC11, respectively. The sequences encoding the HDAC6-like proteins contained two arginase HDAC superfamily domains as identified by both Pfam and the NCBI conserved domains search (Figure 1b). This domain structure is common to HDAC Class IIb proteins. One of the HDAC6-like sequences (XP_016664552) contained an HDAC6-HDAC10 domain 1 and a specific HDAC6 domain 2. HDAC6-HDAC10 domain 1 was also found in the other HDAC6-like sequence (XP_016661953) but an HDAC6 domain 2 was not specifically identified.

3.1.2 | Sirtuins

We also found five sirtuin-related sequences (Sir1, Sir4, Sir5, Sir6, and Sir7—annotated according to their human orthologs) in *A. pisum* and experimentally identified the presence of four of them (Sir1, Sir4, Sir6, and Sir7). We did not detect a Sir5 sequence in our *A. pisum* clone (Figure 2a and Table S1). The phylogenetic tree of sirtuins is comparable to previously published classifications (Frye, 2000; Greiss & Gartner, 2009). The *A. pisum* sirtuin sequences clustered into their corresponding subgroups, with orthologs from the other aphid species (Figure 2a and Figure S2). The *A. pisum* sequence (XP_001943036) annotated in the NCBI database as NAD-dependent HDAC Sir2 clustered in our study in the Sir1 subgroup (Figure 2a). Domain analysis confirmed this finding by identifying the Sir1-specific SIRT1 domain (Figure 2b). We, therefore, refer to this sequence as Sir1 rather than Sir2 (as incorrectly annotated in the NCBI database). We were unable to find additional sirtuin-like sequences (e.g., Sir2 or Sir3) in our *A. pisum* close or in the other aphid species used for phylogenetic analysis (Table S1). Domain analysis revealed Sir2 superfamily domains within the *A. pisum* Sir4, Sir6, and Sir7 sequences, and multiple substrate-binding sites as well as NAD^+ and zinc-binding sites in the proteins Sir1, Sir5, and Sir7 (Figure 2b).

3.1.3 | HATs

Our screen also revealed several members of the GNAT and MYST families. In the GNAT family, we identified one homolog of HAT1, three of KAT2B and one of KAT2A, and in the MYST family, we identified one homolog of KAT5, three of KAT6B, five of KAT7, and three of KAT8 (Figure 3a and Table S1). We experimentally confirmed the presence of one sequence each representing *hat1*, *kat5*, *kat6b*, and *kat7*, three representing *kat2b* and two

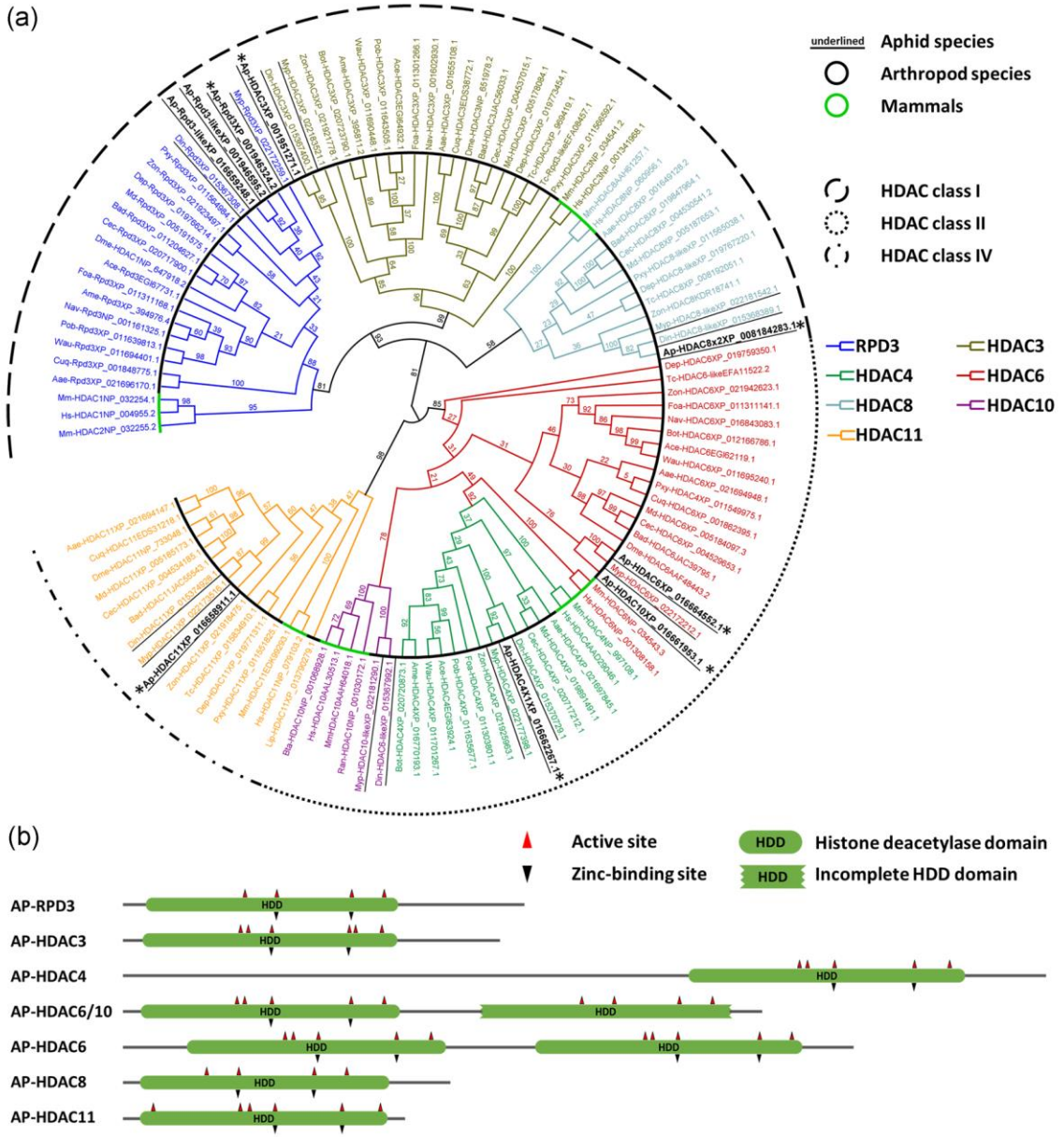

FIGURE 1 Characterization of histone deacetylases (HDACs) in *Acyrthosiphon. pisum.* (a) Phylogeny of HDAC protein sequences. The tree was built with RAxML after MUSCLE alignment using amino acid sequences of HDACs found in *a. pisum* (black and bold) and the orthologs in related aphid species (underlined): *Myzus persicae* (Myp) and *Diuraphis noxia* (Din). The additional homologs are from *Aedes aegypti* (Aae), *Acromyrmex echinatior* (Ace), *Apis mellifera* (Ame), *Bactrocera dorsalis* (Bad), *Bombus terrestris* (Bot), *Bos taurus* (Bta), *Ceratitis capitata* (Cec), *Culex quinquefasciatus* (Cuq), *Dendroctonus ponderosae* (Dep), *Drosophila melanogaster* (Dme), *Fopius arisanus* (Foa), *Homo sapiens* (Hs), *Limulus polyphemus* (Lip), *Musca domestica* (Md), *Melipona quadrifasciata* (Meq), *Mus musculus* (Mm), *Nasonia vitripennis* (Nav), *Pogonomyrmex barbatus* (Pob), *Plutella xylostella* (Pxy), *Rattus norvegicus* (Ran), *Tribolium castaneum* (Tc), *Wasmannia auropunctata* (Wau), and *Zootermopsis nevadensis* (Zon). GenBank accession numbers and bootstrap values are shown within the tree. Experimentally confirmed *A. pisum* LL01 sequences are indicated by asterisks. (b) Protein domains identified in the *A. pisum* HDACs by screening against the Pfam and NCBI conserved domains databases

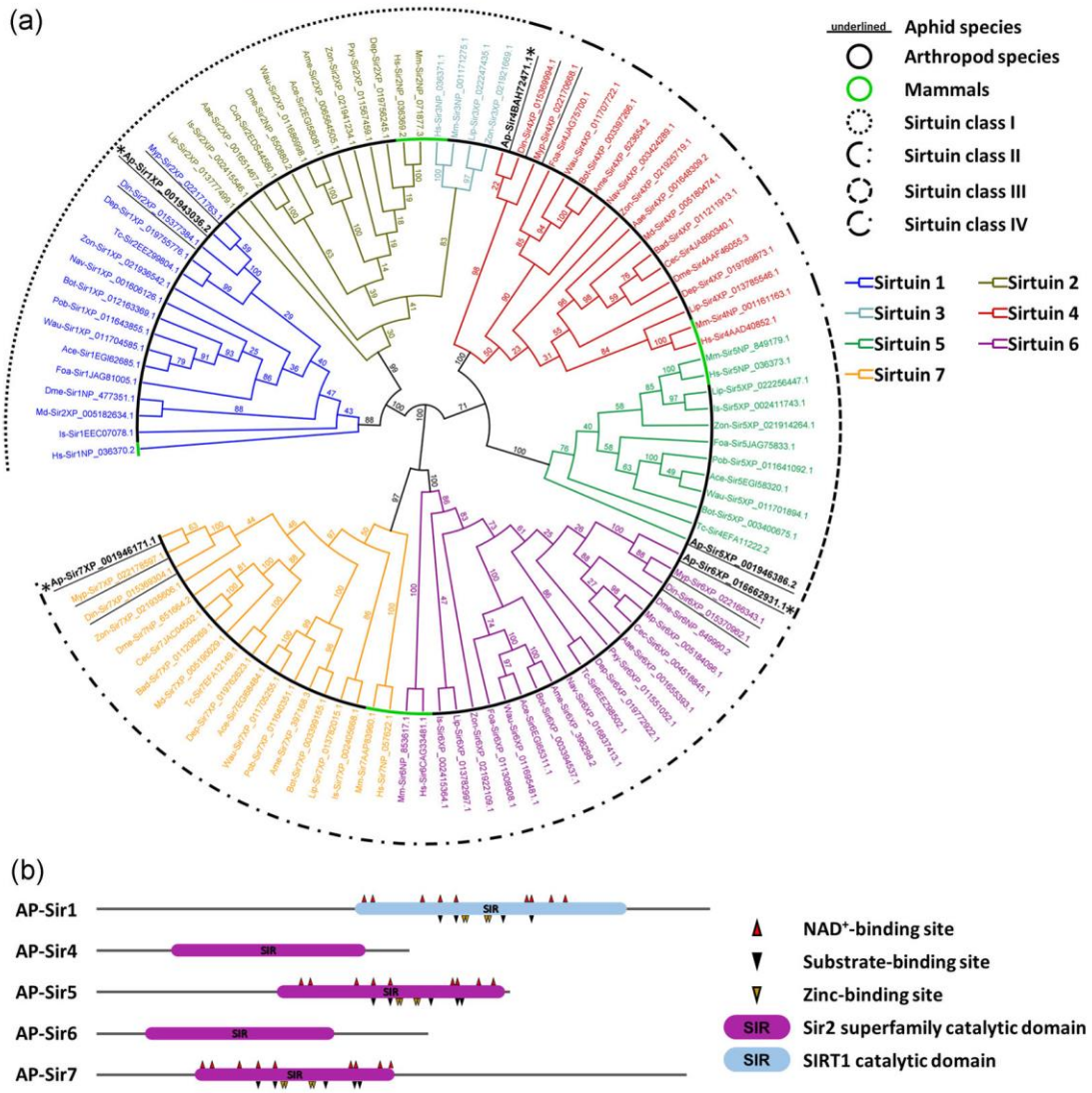

FIGURE 2 Characterization of sirtuins in *A. pisum*. (a) Phylogeny of sirtuin protein sequences. The tree was built with RAxML after MUSCLE alignment using amino acid sequences of sirtuins found in *A. pisum* (black and bold) and the orthologs in related aphid species (underlined): *M. persicae* (Myp) and *D. noxia* (Din). The additional homologs are from *A. aegypti* (Aae), *A. echinatior* (Ace), *A. mellifera* (Ame), *B. dorsalis* (Bad), *B. terrestris* (Bot), *C. capitata* (Cec), *C. quinquefasciatus* (Cuq), *D. ponderosae* (Dep), *D. melanogaster* (Dme), *F. arisanus* (Foa), *Homo sapiens* (Hs), *Ixodes scapularis* (Is), *L. polyphemus* (Lip), *M. domestica* (Md), *M. musculus* (Mm), *N. vitripennis* (Nav), *P. barbatus* (Pob), *P. xylostella* (Pxy), *T. castaneum* (Tc), *Wasmannia suropunctata* (Wau), and *Z. nevadensis* (Zon). GenBank accession numbers and bootstrap values are shown within the tree. Experimentally confirmed *A. pisum* LL01 sequences are indicated by asterisks. (b) Protein domains identified in the *A. pisum* sirtuins by screening against the Pfam and NCBI conserved domains databases

representing *kat8* (Table S1). In the phylogenetic analysis, we excluded heavily fragmented, truncated, incorrectly annotated, and putatively misassembled sequences. Our analysis, therefore, included the HAT1, KAT2A, KAT2B, KAT6B, KAT7, and KAT8 sequences (Figure 3a). The division between the GNAT and MYST families was reproduced in the phylogenetic tree, and all *A. pisum* sequences clustered in the anticipated subgroups (Figure 3a).

Two of the three *A. pisum* KAT2B sequences included a full set of the expected domains (the N-terminal PCAF domain, a GNAT domain, and a BROMO domain), whereas the remaining KAT2B sequence contained a PCAF

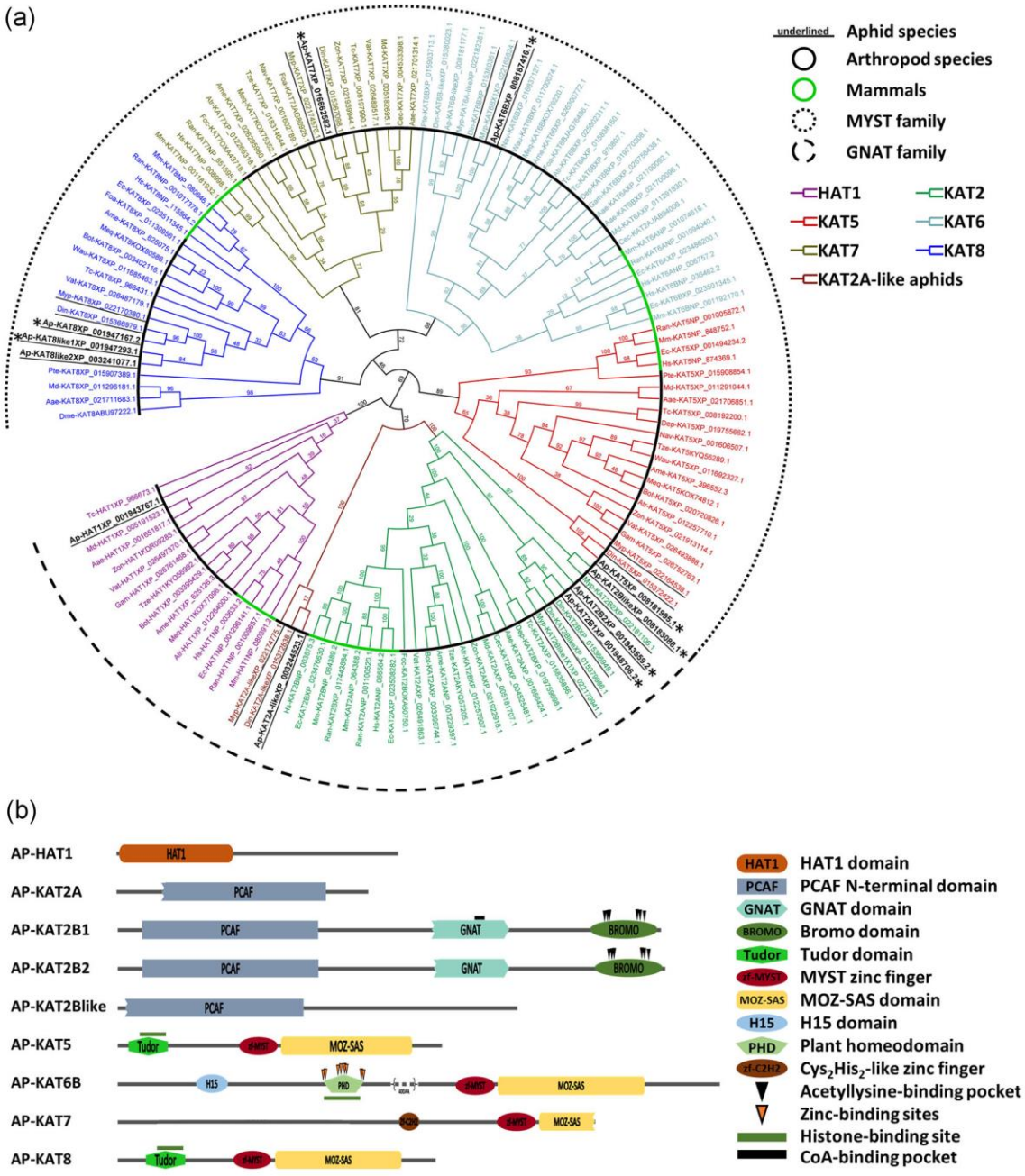

FIGURE 3 Characterization of histone acetylases (HATs) in *A. pisum*. (a) Phylogeny of HAT protein sequences. The tree was built with RAxML after MUSCLE alignment using amino acid sequences of HATs found in *A. pisum* (black and bold) and the orthologs in related aphid species (underlined): *M. persicae* (Myp) and *D. noxia* (Din). The additional homologs are from *A. aegypti* (Aae), *A. mellifera* (Ame), *Athalia rosae* (Atr), *B. terrestris* (Bot), *C. capitata* (Cec), *D. ponderosae* (Dep), *D. melanogaster* (Dme), *Equus caballus* (Ec), *F. arisanus* (Foa), *Folsomia candida* (Foc), Galleria mellonella (Gam), *Homo sapiens* (Hs), *M. domestica* (Md), *M. quadrifasciata* (Meq), *M. musculus* (Mm), *N. vitripennis* (Nav), *Parasteatoda tepidariorum* (Pte), *R. norvegicus* (Ran), *T. castaneum* (Tc), *Vanessa tameamea* (Vat), *W. auropunctata* (Wau), and *Z. nevadensis* (Zon). GenBank accession numbers and bootstrap values are shown within the tree. Experimentally confirmed *A. pisum* LL01 sequences are indicated with asterisks. (b) Protein domains identified in the *A. pisum* HATs by screening against the Pfam and NCBI conserved domains databases. HATs, histone acetyltransferases

domain but lacked the others (Figure 3b). Similarly, the predicted KAT2A sequence (XP_003244523) contained a PCAF domain but no other predicted domains. We were unable to experimentally confirm the presence of *kat2a* in our *A. pisum* clone (Figure 3b). All of the MYST sequences (KAT5, KAT6B, KAT7, and KAT8) contained the characteristic MOZ-SAS domain, and the KAT5 and KAT8 sequences also contained a Tudor domain (Figure 3b). We experimentally confirmed the presence of the two *kat6b* sequences (XP_008187415 and XP_008187416) but further investigation revealed that they comprised the N-terminal (XP_008187416) and C-terminal (XP_008187415) parts of a single KAT6B protein, incorrectly annotated in the NCBI database (see NW_003384476 residues 156881–168587). This *A. pisum* sequence included all domains typical of *kat6b*-like genes, namely the H15, PHP, zf-MYST, and MOZ-SAS domains (Figure 3b). In the KAT7 sequence, we observed a distinctive domain structure comprising a zf-C2H2 motif, as well as zf-MYST and MOZ-SAS domains. However, the MOZ-SAS domain was truncated, suggesting the KAT7 sequence was incomplete (Figure 3b).

3.1.4 | Other genes encoding HDAC and HAT complex subunits

Finally, we also identified and experimentally confirmed one gene encoding an HDAC complex subunit (SAP18) and another encoding KAT14, a major component of the ATAC complex known for its HAT activity (Table S1). In this study, SAP18 and KAT14 as complex subunits were not included in the domain and phylogenetic analysis (Figures 1,3), but only acetylation active enzymes.

3.2 | The effect of silencing HDAC and HAT genes on aphid life-history traits

We evaluated the effect of RNAi targeting aphid HDAC and HAT genes by monitoring survival, development, and reproduction.

For most of the genes encoding HATs (*hat1*, *kat2b1*, *kat2b2*, and *kat8*) and HDACs (*hdac3*, *hdac6*, *hdac8*, *sap18*, and *sir1*), silencing did not affect the monitored life-history traits in *A. pisum* (Figures S3–S6 and Tables S4–S5). Mild effects were induced by silencing *kat6b* or *kat7*. Aphids injected with *kat7* dsRNA produced more offspring, whereas those injected with *kat6b* dsRNA survived for longer than the GFP control group (Figures S5 and S7; Table S4). RNAi-mediated suppression of *kat14* extended the lifespan of aphids by ~4 days, probably explaining the higher number of offspring (Figure 4a,c and Table S4).

Silencing the HDAC gene *rpd3* did not affect aphid survival, but reproduction ceased ~5 days earlier than in the GFP control group (Figure 4f black box and Table S5). Aphids injected with *rpd3* dsRNA also produced premature offspring, but there was no significant difference in the total number of offspring between the *rpd3* dsRNA group and controls (Figure 4d and Table S4). The premature offspring indicated that *rpd3* plays a role in embryogenesis and/or eclosion.

Silencing the genes encoding HDACs and HATs did not induce other developmental changes (e.g., deformations), polyphenism (e.g., sexual/asexual, winged/wingless) or changes in body color or size.

3.3 | Effect of chemical inhibitors on life-history traits of aphids

Next, we tested the effect of the HDAC inhibitor SAHA and the HAT inhibitor epi-ML on the same aphid life-history traits monitored in the RNAi experiments. The effect of each compound was determined by tracking aphid survival during 5 days of feeding (Figure 5). Both inhibitors showed dose-dependent insecticidal activity against *A. pisum* at low, medium, and high concentrations (10, 50, and 150 µg/ml for SAHA; 5, 10, and 25 µg/ml for epi-ML). The high concentrations reduced aphid survival by 70% (SAHA) and ~50% (epi-ML), whereas the medium and low concentrations exhibited milder but still significant effects on survival (Figure 5).

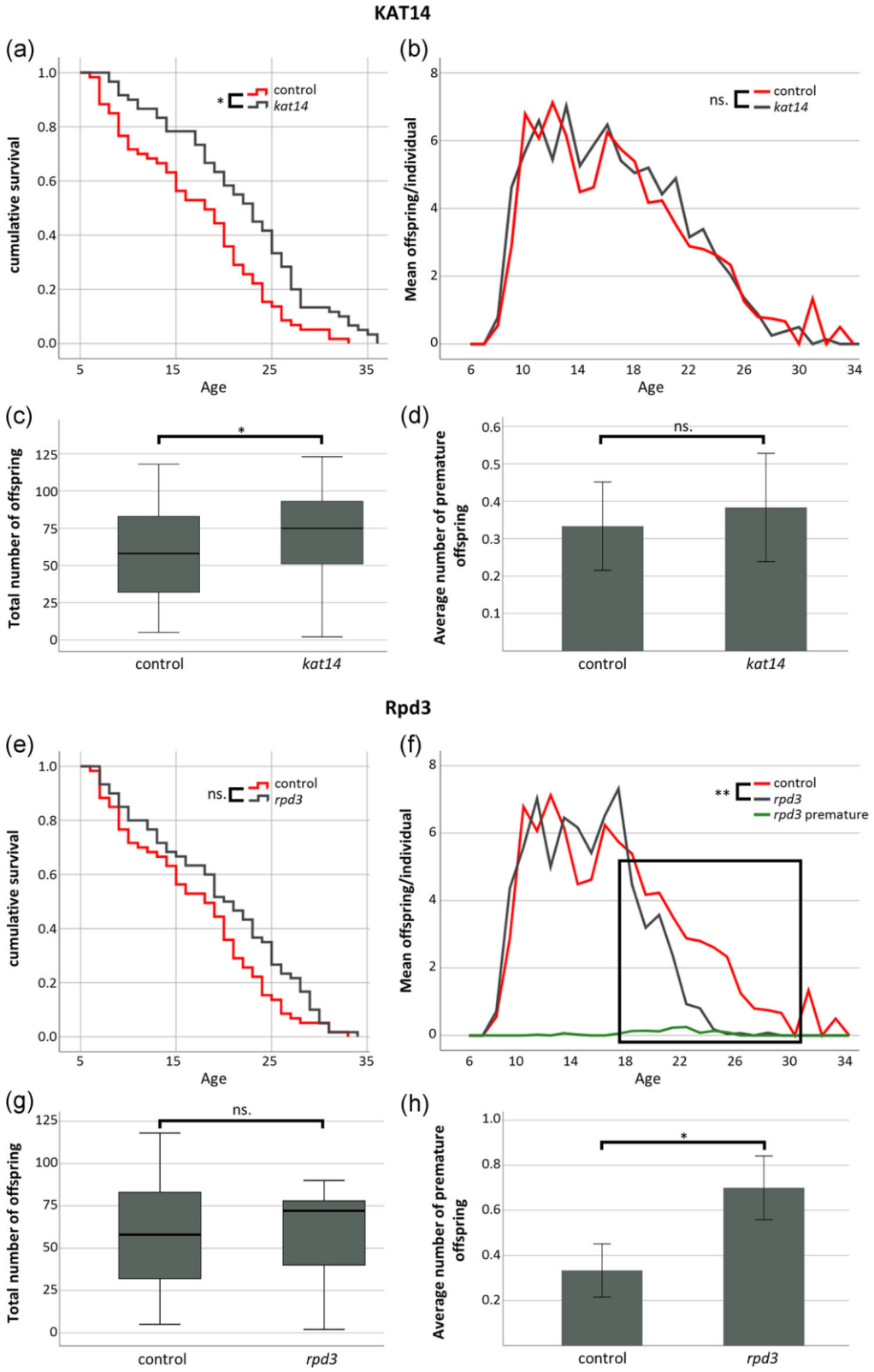

FIGURE 4 Continued.

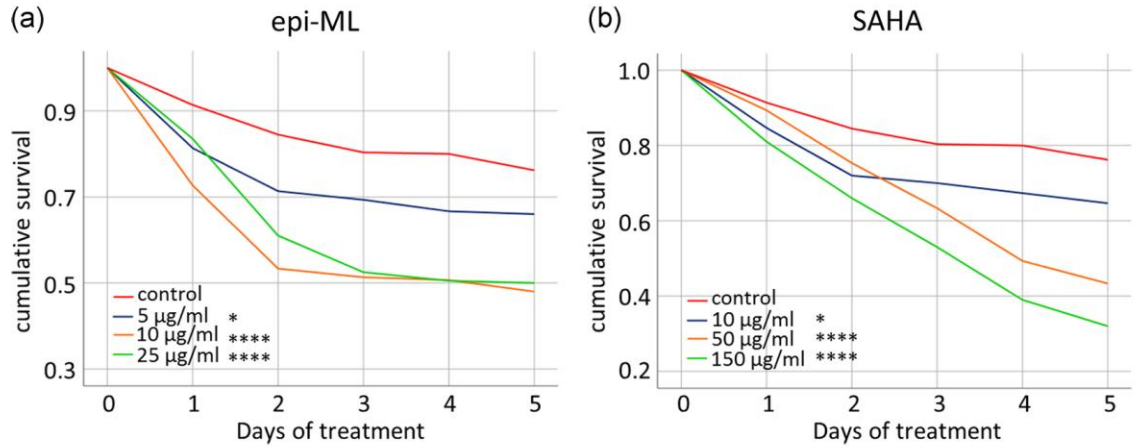

FIGURE 5 Effect of the inhibitors epi-ML and SAHA on the survival of *A. pisum*. Aphid survival was monitored during 5 days of exposure to AP3 diet mixed with the HAT inhibitor epi-ML or the HDAC inhibitor SAHA. Survival data were analyzed using Kaplan–Meier and logrank tests. The AP3 diet (plain or with solvent) was used as a negative control. (a) HAT inhibitor epi-ML. (b) HDAC inhibitor SAHA. Statistical significance is indicated by asterisks: $^{*}p < .05$, $^{****}p < .0001$. HAT, histone acetyltransferase; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid

Interestingly, epi-ML caused a more significant decline in aphid survival during the first 2–3 days of exposure, whereas the same profile was observed at the low concentration of SAHA, but not at the medium and high concentrations, where the decrease in survival was nearer to linear. Aphids that survived for 5 days were monitored for a further 10 days to detect any delayed effects of the epigenetic inhibitors. In most cases, there was no significant difference in survival compared to the corresponding control treatment. However, the survival rate continued to decline in the aphid population exposed to the high concentration of SAHA (Figure S7).

To determine the effect of epigenetic inhibitors on aphid reproduction and development, we monitored several parameters daily for a period of 10 days after the treatments were completed: the start of reproduction, the total number of offspring, the number of offspring per day, the body size, and the body weight (Figure 6). Aphid reproduction and development were affected by both inhibitors. High concentrations of epi-ML caused significant reproductive delays of more than 1–2 days compared to the control treatment (Figure 6a). Moreover, aphids exposed to epi-ML also produced significantly fewer offspring in total (Figure 6b). The high concentration of SAHA did not cause a reproductive delay, but significantly reduced the total number of offspring. Interestingly, both the medium and low concentrations of SAHA negatively affected all reproductive parameters (Figure 6f,g). In the group of aphids exposed to the HAT inhibitor epi-ML, the body size was significantly smaller and the body weight significantly lower compared to controls (Figure 6c,d). In contrast, aphids exposed to HDAC inhibitor SAHA were slightly heavier than untreated controls, but there was no difference in body size (Figure 6h,i).

FIGURE 4 RNAi-mediated silencing of *A. pisum* KAT14 (HAT) and Rpd3 (HDAC). Several life-history parameters were monitored in aphids injected with dsRNA to suppress KAT14 (a–d) and Rpd3 (e–h) and were compared to control injected with dsRNA matching the irrelevant gene encoding GFP. The following life-history traits were monitored: survival (a, e), number of offspring per day (b, f), the total number of offspring (c, g) and the total number of premature offspring (d, h). Survival data were shown with Kaplan–Meier plot and tested for significances by logrank test. The number of normal and premature offspring (total or per day) was analyzed by ANOVA, Student's *t* test and the Mann–Whitney *U* test. Significant differences in daily reproduction between the Rpd3 and GFP control groups are highlighted in a black square where the threshold was $p < .001$ for ANOVA. Statistical significances are indicated by asterisks: $^{*}p < .05$, $^{**}p < .0001$, *ns* = not significant ($p > .05$). ANOVA, Analysis of variance; HAT, histone acetyltransferase; HDAC, histone deacetylase; GFP, green fluorescent protein; RNAi, RNA interference

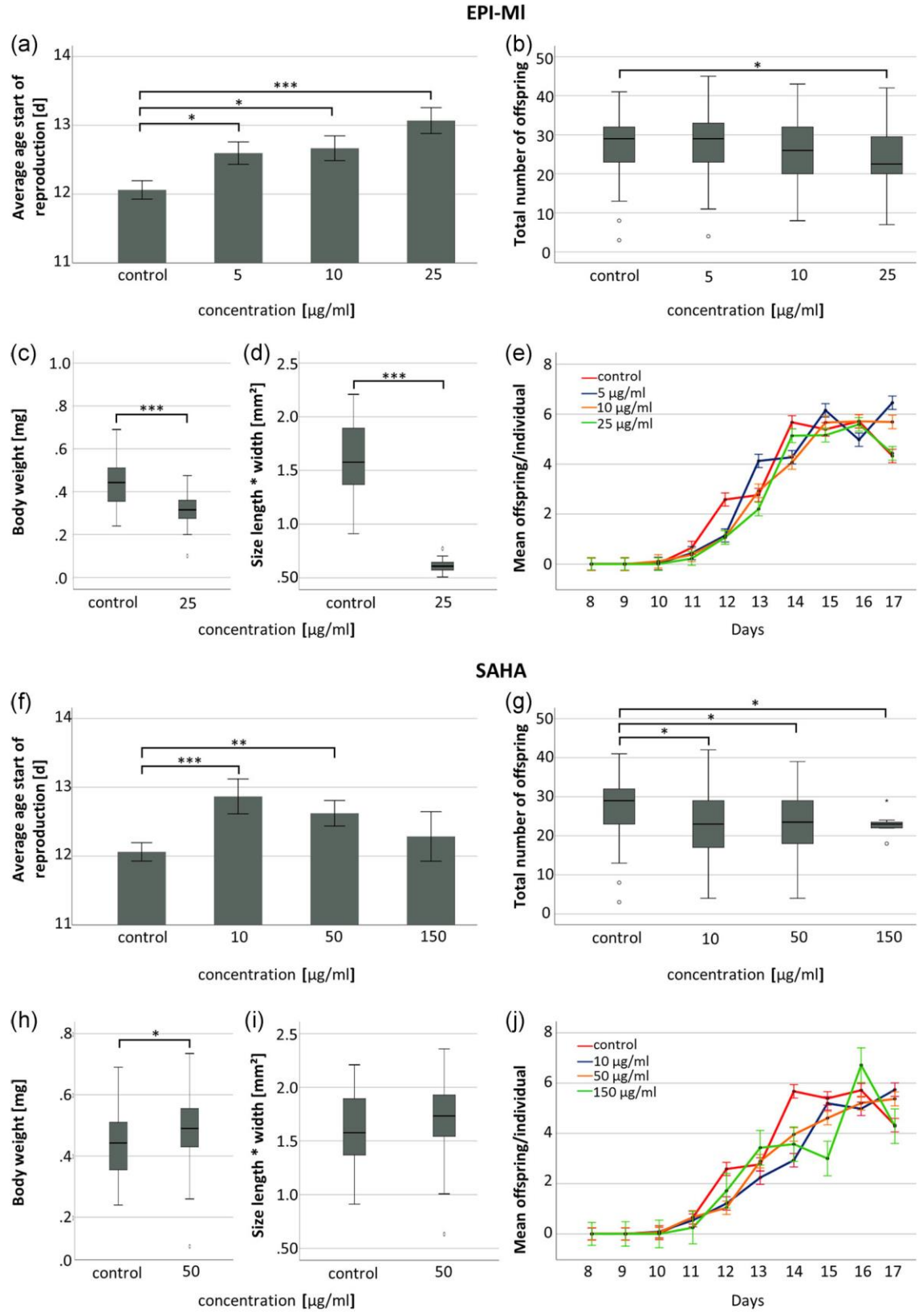

FIGURE 6 Continued.

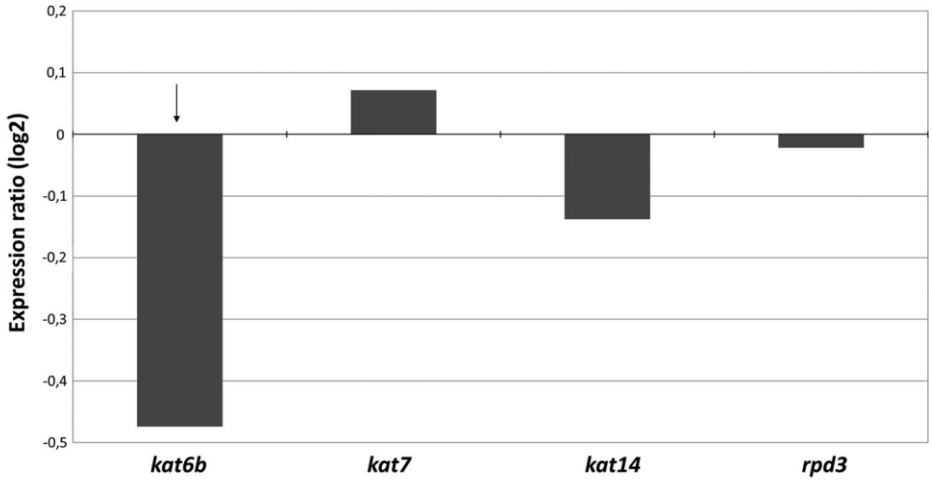

FIGURE 7 RNA-mediated knockdown in *A. pisum* 2 days after the delivery of gene-specific dsRNA. Expression values were normalized against the reference genes *rpl32* and *actin*. Negative expression ratios indicate downregulation and positive ratios indicate upregulation. Arrow indicates the significant modulation of gene expression as determined by REST analysis ($p < .05$). dsRNA, double-strand RNA

3.4 | Effect of RNAi and chemical inhibitors on gene expression in *A. pisum*

We used qPCR to investigate the expression levels of genes involved in histone acetylation/deacetylation following the injection of aphids with the corresponding dsRNAs or feeding with the inhibitors epi-ML or SAHA (Figures 7 and 8; Tables S6 and S7). Gene expression was measured in the whole body or in different isolated parts (head, gut, embryos and the residual body tissue, described here as the carcass). Gene expression was measured only for RNAi targets *kat6b*, *kat7*, *kat14*, and *rpd3*, which showed an effect when silenced (Figure 4; Tables S4 and S5). We observed the significant downregulation of *kat6b* (0.5-fold) 48 hr postinjection, but there was no effect on the other genes encoding HATs (*kat7* and *kat14*) or HDACs (*rpd3*; Figure S7).

The orally delivered epigenetic inhibitors SAHA and epi-ML also modulated the expression levels of various genes involved in histone acetylation/deacetylation (Figure 8). The inhibition of HATs by epi-ML caused the upregulation of *kat14* 1.5–2.3-fold) and *hat1* 2.8–4.5-fold) in all examined tissues (Figure 8a and Table S6). Furthermore, *kat8* was upregulated (1.8-fold) in the carcass, whereas *kat6b* was downregulated (0.4-fold) in embryos. The inhibition of HDACs by SAHA upregulated the expression of *kat14* (2.2-fold) and *kat8* (1.8-fold) in the head, but no significant changes were observed elsewhere. The expression of *hat1* was only significantly upregulated in the head (2.7-fold) but there was a trend toward increased expression in all other tissues. SAHA caused the downregulation of *kat6b* (0.3-fold), *rpd3* (0.4-fold), and *hdac8* (0.4-fold) in embryos (Figure 8b and Table S7). Furthermore, both inhibitors suppressed the expression of *kat2b1*, although the only statistically significant effect was the suppression of *kat2b1* in the gut (0.7-fold) by epi-ML (Figure 8; Tables S6 and S7).

FIGURE 6 Effect of the HAT inhibitor epi-ML and the HDAC inhibitor SAHA on life-history traits in the aphid *A. pisum* compared to an untreated control group. (a–e) EPI-MI treatment. (f–j) SAHA treatment. The following life-history traits were monitored: the average start of reproduction (a, f), total number of offspring (b, g), number of offspring per day (e, j), body weight (c, h), and body size (d, i). The reproductive parameters were determined for the three concentrations of both inhibitors (indicated in μg/ml), whereas body weight and body size were evaluated only for the highest concentration of epi-ML and sublethal concentration of SAHA. The start of reproduction data was analyzed by logrank test, whereas ANOVA and Student's *t* test were used for data analysis of the reproductive parameters as well as body weight and body size. Statistical significances are indicated by asterisks: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. ANOVA, Analysis of variance; HAT, histone acetyltransferase; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid

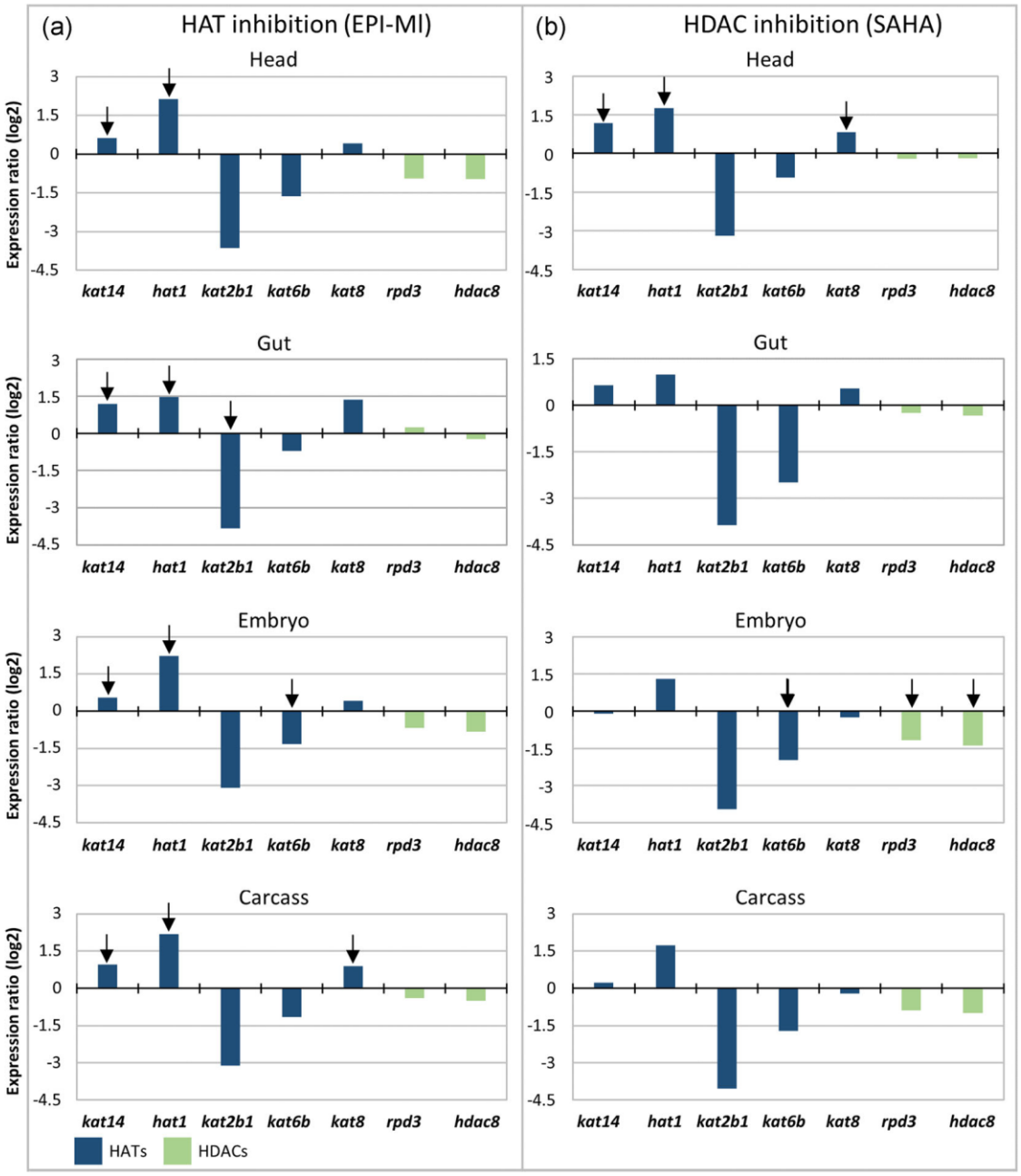

FIGURE 8 Expression of histone acetylation/deacetylation associated genes in *A. pisum* after treatment with the HAT inhibitor epi-ML or the HDAC inhibitor SAHA. Data show gene expression in representative tissues (head, gut, embryos, and carcass) after exposure to epi-ML (25 µg/ml; a) or SAHA (50 µg/ml; b) for 5 days. Values were normalized against the reference gene rpl32. Negative expression ratios indicate downregulation and positive ratios indicate upregulation. HAT genes are indicated in blue and HDAC genes are indicated in green. Arrows indicate the significant modulation of gene expression as determined by REST analysis ($p < .05$). HAT, histone acetyltransferase; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid

4 | DISCUSSION

Lysine acetylation is a key regulatory mechanism in all eukaryotes, with important roles not only in gene regulation but also cell signaling and metabolism (Ali et al., 2018). Previous studies have shown that the aphid *A. pisum* has an unusually large number of genes involved in chromatin remodeling by acetylation, methylation, and ubiquitination compared to other arthropods (Rider et al., 2010; The International Aphid Genomics Consortium, 2010). We have confirmed this diversity for the various families of HATs and HDACs by identifying *A. pisum* enzymes that phylogenetically cluster into all known enzyme classes (Figures 1–3). Such diversity may reflect recent gene duplication and divergence events in *A. pisum* (Duncan, Feng, Nguyen, & Wilson, 2016; Duncan, Leask, & Dearden, 2013; Gilbert, 2009; Rider et al., 2010; The International Aphid Genomics Consortium, 2010). For example, the *D. melanogaster* genome encodes six GCN5 family and four MYST family HATs, compared to five GCN5 family and 12 MYST family HATs in our *A. pisum* clone (Rider et al., 2010). Similarly, we identified two HDAC8 and four Rpd3 sequences in *A. pisum*, whereas duplications of these genes are uncommon in other arthropod lineages (Rider et al., 2010)

Assembly and annotation errors in draft genomes can lead to a significant overestimation of gene diversity, often reflecting the misidentification of allelic variants as separate paralogous loci (split genes) or the fragmentation of genes onto multiple contigs or scaffolds (cleaved genes; Denton et al., 2014). The *A. pisum* genome annotation provides evidence for split genes (e.g., *hdac8* and *rpd3*) and cleaved ones (e.g., *kat6b* and *kat7*). Although we confirmed that the repertoire of genes encoding histone-modifying enzymes in *A. pisum* has expanded (e.g., *kat2b1* and *kat2b2*), earlier reports may have overestimated gene diversity due to the quality of the draft genome available at the time (Denton et al., 2014; Rider et al., 2010). We also found two HDAC Class IIb sequences, clustering together in the HDAC6 group, as well as HDAC11 (Class IV HDAC), which was initially not found in *A. pisum* (Rider et al., 2010; Figure 1 and Table S1). Our study also showed that the *A. pisum* sequence identified as Sir1 was originally annotated incorrectly as the NAD-dependent HDAC Sir2 (Figure 2).

The characterization of genes encoding HATs and HDACs enabled us to use RNAi to investigate the functions of those genes, a technique that has been applied in many insect species including aphids (Abdellatef et al., 2015; Knorr et al., 2018; Will et al., 2017; Ye et al., 2019). We found that the silencing of KAT6B and KAT14 significantly extended the aphid lifespan (Figure 4a and Figure S5A), agreeing with earlier reports in *D. melanogaster* that the midlife silencing of KAT7 (also known as Chameau) increases longevity, suggesting that the loss of acetylation may help to balance gene expression in aged insects (Peleg, Feller, Forne et al., 2016; Peleg, Feller, Ladurner, & Imhof, 2016; Solovev, Shaposhnikov, Kudryavtseva, & Moskalev, 2018). Similarly, increasing the activity of deacetylases such as Sir2 and SIRT6 promotes survival in several organisms including yeast, *D. melanogaster*, *Caenorhabditis elegans* and mice (Benayoun, Pollina, & Brunet, 2015; Dang, 2014). We will investigate the effects of HAT/HDAC overexpression in future studies, as well as the impact of HAT silencing in aged aphids.

As well as promoting survival, the silencing of KAT14 increased the number of aphid offspring (Figure 4c). This probably reflects the extension of the reproductive phase in the treated aphids as a consequence of their prolonged survival. In contrast, the silencing of KAT7 expression did not affect longevity, but the aphids nevertheless produced significantly more offspring than the controls (Figure S5C). In *D. melanogaster*, KAT7 controls DNA replication and affects longevity (McConnell, Dixon, & Calvi, 2012; Peleg, Feller, Forne et al., 2016). The mechanism by which this protein influences reproduction in aphids is unclear.

Silencing the HDAC Rpd3 did not have a significant impact on aphid survival (Figure 4e and Table S4) but these aphids produced prematurely born offspring (Figure 4f,h). Embryos of fruit flies lacking maternally expressed *rpd3* failed to hatch and had segmentation defects (Chen, Fernandez, Mische, & Courey, 1999). Therefore, our results suggest that Rpd3 has an important role in aphid embryogenesis and eclosion.

As previously mentioned, silencing of the majority of gene targets (HATs: *hat1*, *kat2b1*, *kat2b2*, and *kat8*; and HDACs: *hdac3*, *hdac6*, *hdac8*, *sap18*, and *sir1*) did not induce any effect on parameters monitored in aphids. In agreement with our data, a growing number of studies report variable RNAi efficiencies in hemipterans ranging

from no phenotype to significant effects on survival (Joga, Zotti, Smagghe, & Christiaens, 2016; Singh et al., 2017). This is frequently correlated with dsRNA degradation caused by gut, saliva, and hemolymph associated nucleases, but also upon the nature of the gene target (Cao, Gatehouse, & Fitches, 2018; Christiaens et al., 2014; Singh et al., 2017). Follow-up studies should investigate whether the silencing of "nonactive" genes affects some other traits in aphids that were not monitored in this study.

As well as inhibiting the expression of specific HAT and HDAC genes by RNAi, we also fed aphids on chemicals that inhibited the activity of HATs or HDACs generally. In each case, this had a severe impact on aphid survival (Figure 5). Both inhibitors to some extent delayed the start of reproduction and reduced the number of offspring even at low concentrations (Figure 6a,b,f,g). The inhibition of HATs using epi-ML reduced the aphid body size and body weight (Figure 6c,d), whereas the inhibition of HDACs using SAHA significantly increased the mean body weight without affecting body size (Figure 6h). In agreement with our data, the inhibition of HDACs also reduced survival and caused developmental arrest in *D. melanogaster* (Pile et al., 2001). Similar effects were observed in the frog *Xenopus laevis* and the starfish *Asterina pectinifera* (Almouzni, Khochbin, Dimitrov, & Wolffe, 1994; Ikegami et al., 1993; Tseng, Carneiro, Lemire, & Levin, 2011). When we exposed aphids to SAHA, we found that the inhibition of development was indicated by a delayed reproductive onset, but the treated aphids nevertheless achieved a greater body weight than the untreated control cohort, probably reflecting nonspecific cell proliferation similar to that observed in *X. laevis* (Sachs, Amano, Rouse, & Shi, 2001). We saw no evidence that the inhibition of HDAC activity extended the lifespan of the aphids, even though this phenomenon was previously observed in fruit flies (Kozer, etska, Serga, Koliada, & Vaiserman, 2017) albeit not in all studies (Pile et al., 2001). Such differences between studies are likely to reflect the experimental setup, the age of the insects during treatment, the duration of exposure, and the genotype of the experimental subjects. Future studies of epigenetic factors should control for genetic and environmental effects to ensure the generation of reproducible results.

A. pisum is a model for the analysis of phenotypic plasticity, but we observed no polyphenetic responses to the dsRNA injections or exposure to the chemical inhibitors used in this study. The application of Zebularine and RG108 (inhibitors of DNA methylation) to *A. pisum* induced the appearance of wings, indicating that polyphenism has an epigenetic component (Dombrovsky, Arthaud, Ledger, Tares, & Robichon, 2009). Although histone acetylation does not appear to influence this phenomenon, it is possible that other forms of histone modification may play a role. Several studies suggest that epi-ML is not only able to inhibit HATs, but also lysine methyltransferases (Mai et al., 2008). It may be that the epi-ML associated effects in aphids are partially also associated with inhibition of methyltransferases. Follow-up studies should investigate the correlation between epi-ML and inhibition of histone methyltransferases in aphids.

Finally, we investigated the expression of genes encoding HATs and HDACs in *A. pisum* following dsRNA injection or exposure to the chemical inhibitors (Figures 7 and 8; Tables S6 and S7). We anticipated that dsRNAs would have a direct impact on the expression of target genes at the posttranscriptional level, but both RNAi and the chemical inhibitors also have the potential to modulate transcription by means of feedback regulation to maintain chromatin homeostasis (Peserico & Simone, 2011). Although the knockdown of *kat7*, *kat14*, *rpd3*, and *kat6b* caused significant effects on monitored life-history traits in *A. pisum*, only the *kat6b* knockdown achieved a corresponding direct loss of mRNA (Figure 7). Due to variable RNAi efficiencies that may occur in hemipteran insects, it is possible that RNAi phenotype can be observed, but there is no evidence for gene knockdown (Cao et al., 2018). The injection of dsRNA can lead to the degradation of mRNA and/or the inhibition of translation, which may explain the outcome of the *kat7*, *kat14*, and *rpd3* knockdown experiments, where mRNA levels appeared normal. It is possible that protein levels were lower in these insects due to the inhibition of translation, and this would require the quantitation of each protein by western blot or similar methods (Holmes, Williams, Chapman, & Cross, 2010).

We found that the chemical inhibition of HATs had no effect on HDAC gene expression whereas the chemical inhibition of HDACs modulated the expression of HAT genes such as *kat8*, *kat14*, *hat1*, and *kat6b* (Figure 8). In *G. melonella*, Mukherjee et al. (2012) found that the genes encoding HATs and HDACs were controlled by feedback regulation following exposure to epi-ML and SAHA. We did not observe such clear feedback regulation in *A. pisum*. Instead, SAHA appeared to induce stress in the aphids, triggering changes in the expression of HAT genes involved

in stress responses. Further proteomic studies are required to determine the tissue-specific activities of each HAT or HDAC in aphids and their related changes triggered by chemical inhibition with SAHA and epi-ML. This may contribute to our better understanding of the function of specific HATs and HDACs in aphid tissues.

In summary, we have characterized the genes involved in histone acetylation/deacetylation in *A. pisum* and determined their phylogenetic relationships with other organisms. We evaluated changes in aphid life-history traits triggered by HAT/HDAC inhibition. This study significantly contributes to our understanding of aphid enzymes involved in the regulation of histone acetylation as a major posttranslational modification. It would be valuable to investigate whether nonhistone proteins were affected by HAT/HDAC inhibition used in this study and how this influences life-history traits of aphids.

We did not observe clear correlations between the two experimental approaches (RNAi and feeding with chemical inhibitors), probably because the impact of RNAi is restricted to the gene or genes matching the dsRNA construct whereas epi-ML and SAHA inhibit entire enzymatic classes. The chemical inhibitors, therefore, exert an effect that represents the combined impact of targeting all the individual genes. Future studies should investigate the simultaneous knockdown of multiple aphid genes that may coregulate histone acetylation/deacetylation as recently shown in *D. melanogaster* (McConnell et al., 2012). It would thus be possible to induce stronger effects on aphids than the knockdown of single genes. This will set the foundation for the identification of genes that can be targeted by RNAi to protect plants from the severe agricultural damage caused by aphid pests.

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AUTHOR CONTRIBUTIONS

P.K. and M.S. (Marisa Skaljac) designed the study, analyzed the data, and drafted the manuscript. P.K., J.G., T.K., M.S. (Maximilian Seip), and K.M. performed the experiments. A.V. conceived and supervised the study, revised the manuscript and contributed reagents, materials, and analytical tools. All authors are accountable for the final content of the manuscript and approved its publication.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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5.2 2ND PUBLICATION

2 Lysine acetyltransferase p300/CBP plays an important role in reproduction, embryogenesis and longevity of the pea aphid *Acyrthosiphon pisum*

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Lysine Acetyltransferase p300/CBP Plays an Important Role in Reproduction, Embryogenesis and Longevity of the Pea Aphid *Acyrthosiphon pisum*

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Abstract: CREB-binding protein (p300/CBP) is a universal transcriptional co-regulator with lysine acetyltransferase activity. *Drosophila melanogaster* p300/CBP is a well-known regulator of embryogenesis, and recent studies in beetles and cockroaches have revealed the importance of this protein during post-embryonic development and endocrine signaling. In pest insects, p300/CBP may therefore offer a useful target for control methods based on RNA interference (RNAi). We investigated the role of p300/CBP in the pea aphid (*Acyrthosiphon pisum*), a notorious pest insect used as a laboratory model for the analysis of complex life-history traits. The RNAi-based attenuation of *A. pisum* p300/CBP significantly reduced the aphid lifespan and number of offspring, as well as shortening the reproductive phase, suggesting the manipulation of this gene contributes to accelerated senescence. Furthermore, injection of p300/CBP dsRNA also reduced the number of viable offspring and increased the number of premature nymphs, which developed in abnormally structured ovaries. Our data confirm the evolutionarily conserved function of p300/CBP during insect embryogenesis and show that the protein has a critical effect on longevity, reproduction and development in *A. pisum*. The potent effect of p300/CBP silencing indicates that this regulatory protein is an ideal target for RNAi-based aphid control.

Keywords: KAT3; CREB-binding protein; RNA interference; senescence; life-history traits; *nejire*

1. Introduction

Protein acetylation in eukaryotes is a major post-translational modification, in which acetyl coenzyme A acts as an acetyl group donor [1,2]. Although discovered as a unique modification of histones, acetylation marks are found on numerous non-histone proteins in all cellular compartments [3,4]. The acetylation of proteins regulates many processes, including gene expression, cell cycle progression, development and aging [3,4]. Acetylation affects the function of proteins by conferring a positive charge, which influences stability, enzymatic activity, subcellular localization and cross-talk with other protein modifications such as methylation [4].

The acetylation of proteins is regulated by the opposing activity of lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) [3,5]. KATs catalyze the transfer of acetyl groups to a lysine residue, whereas KDACs remove these groups [5]. A fine balance between KAT and KDAC activities maintains normal biologic functions [6], so any disruption of this balance (caused naturally or triggered by the use of inhibitors) can severely affect physiology and development [7–9].

There is a remarkably diverse panel of highly conserved KDACs and KATs in many organisms [3]. Eleven groups of KDACs have been defined (KDAC1–KDAC11), whereas most KATs are assigned to three groups: the GCN5-related N-acetyltransferases (GNAT family); the p300/CREB-binding proteins (p300/CBP family); and the MOZ/Ybf2/Sas2/Tip60 (MYST) family [3]. The paralogs p300 (also known as EP300 and KAT3B) and CBP (also known as CREBBP, KAT3A and *nejire*) are often collectively described as p300/CBP [10,11].

In higher eukaryotes, p300/CBP is a key transcriptional co-regulator of basic cellular functions [10–12]. Evolutionary studies have identified p300/CBP as an essential enzyme that regulates the growth and development of multicellular organisms by controlling cell-to-cell signaling and morphogenesis [10,13–15]. Furthermore, p300/CBP is a major component of multiple signaling pathways [16–19]. More than 400 p300/CBP target proteins have been identified, leading to the acetylation of ~100 protein substrates [10]. The dysregulation of p300/CBP has been associated with several human diseases, including various forms of cancer [20]. In *Drosophila melanogaster*, the loss of p300/CBP activity causes severe embryonic defects [21,22]. In the cockroach *Blatella germanica* and the red flour beetle *Tribolium castaneum*, the knockdown of this gene revealed multiple roles in postembryonic development [23–25].

The pea aphid (*Acyrthosiphon pisum*) is a laboratory model for the analysis of plant–insect interactions and complex life-history traits and was the first hemipteran insect with a complete published genome sequence [26–28]. It is also a pest insect that damages crops by direct feeding and by vectoring numerous plant viruses [29]. A comprehensive set of *A. pisum* acetylation enzymes has been identified, some of which (KAT6B, KAT7, KAT14 and RPD3) regulate life-history traits such as longevity, development and reproduction [30,31]. Although histone acetylation may induce reproductive and wing morphology polyphenism in some aphids, no such correlation has been identified in *A. pisum* [30,32–34]. Despite the central role of p300/CBP as a transcriptional co-regulator, nothing is yet known about the function of this protein in aphids.

To address this knowledge gap, we investigated the role of p300/CBP in *A. pisum* by RNA interference (RNAi), a powerful approach for the functional analysis of genes in insects [35–38]. RNAi can also be used as a pest control strategy, by expressing double-stranded RNA (dsRNA) in crops or applying it as sprays [38–45]. We injected aphids with *p300/CBP* dsRNA and measured their fitness parameters to determine the effect of RNAi-mediated p300/CBP attenuation on longevity, reproduction and embryogenesis.

2. Materials and Methods

2.1. Aphid Rearing

A. pisum parthenogenetic clone LL01 was reared on 2–3-week-old bean plants (*Vicia faba* var. minor) in a KBWF 720 climate cabinet (Binder, Tuttlingen, Germany) with a 16-h photoperiod and a day/night temperature regime of 24/18 °C [37,46].

2.2. RNA Extraction, Target Gene Identification

We extracted total RNA from pools of 10 aphids using the NucleoSpin RNA kit (Macherey–Nagel, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized from 100 ng RNA using the RevertAid first strand cDNA synthesis kit and dT primers (Thermo Fisher Scientific, Dreieich, Germany). We sequenced nine overlapping fragments covering the open reading frame (ORF) together with the 5′ untranslated region (5′-UTR) of the *A. pisum p300/CBP* mRNA (Figure 1, Table S1). The primers for sequencing were designed using Primer3 v4.1.0 (http://primer3.ut.ee/) and were based on the *A. pisum* sequence template from the NCBI database (XM_003242184). The overlapping p300/CBP fragments were cloned and sequenced as previously described [47].

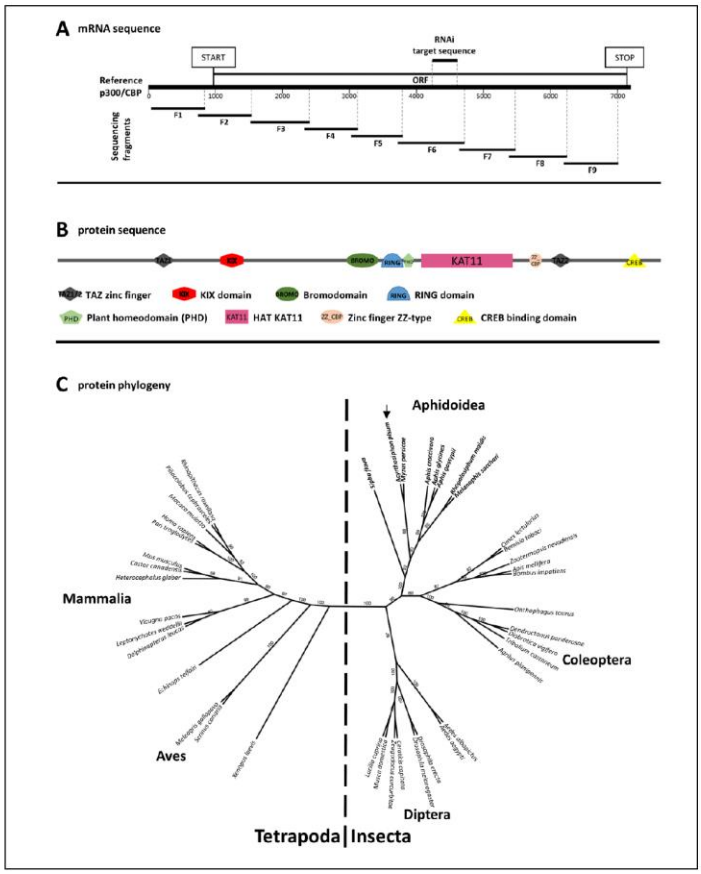

Figure 1. Characteristics of the *p300/CBP* sequences used in this study. (**A**) The *A. pisum p300/CBP* mRNA reference sequence (XM_008188962) is shown, the location of the open reading frame (ORF) as well as the RNAi target site is indicated. The orientation of the nine fragments obtained by cloning and Sanger-sequencing (Supplementary Fragments 1–9, Table S1) is depicted. These fragments were used for the assembly of *A. pisum* p300/CBP sequence. Our assembly contains the 5′-UTR and most of the open reading frame (ORF) including the start codon, but not the stop codon and 3′-UTR (**B**) Domain analysis of the p300/CBP protein sequence using Pfam and NCBI conserved domains databases. A complete set of p300/CBP typical domains was identified (**C**) Phylogeny of p300/CBP protein sequences. The tree was generated with RAxMl after MUSCLE alignment using amino acid sequence of *A. pisum* (black arrow/XP_003242232), *Sipha flava* (XP_025414151), *Myzus persicae* (XP_022176157), *Aphis craccivora* (KAF0769549), *Aphis glycines* (KAE9537982), *Aphis gossypii* (XP_027838800), *Rhopalosiphum maidis* (XP_026820749), *Melanaphis sacchari* (XP_025193438), *Cimex lectularius* (XP_014253865), *Bemisia tabaci* (XP_018901305), *Zootermopsis nevadensis* (XP_021919144), *Apis mellifera* (XP_026294862), *Bombus impatiens* (XP_012242677), *Onthophagus taurus* (XP_022908965), *Dendroctonus ponderosae* (XP_019756971), *Diabrotica vigifera* (XP_028149091) *Tribolium castaneum* (XP_008192360), *Agrilus planipennis* (XP_025830621), *Aedes albopictus* (XP_029711694), *Aedes aegypti* (XP_011493407), *Drosophila erecta* (XP_015011063), *Drosophila melanogaster* (NP_524642), *Ceratitis capitata* (XP_012155269), *Zeugodacus curcurbitae* (XP_028900992), *Musca domestica* (XP_011290197), *Lucilia cuprina* (XP_023298299), *Xenopus laevis* (NP_001088637), *Serinus canaria* (XP_009084782), *Meleagris gallopavo* (XP_010710456), *Echinops telfairi* (XP_004700331), *Delphinapterus leucas* (XP_022452845), *Leptonychotes weddellii* (XP_006729983), *Vicugna pacos* (XP_006207247), *Heterocephalus glaber* (EHB13435), *Castor canadensis* (JAV39871), *Mus musculus* (NP_808489), *Pan troglodytes* (NP_001231599), *Homo sapiens* (AAA18639), *Macaca mulatta* (NP_001253415), *Piliocolobus tephrosceles* (XP_023077657), *Rhinopithecus roxellana* (XP_010375568). Defined organism family clusters are indicated. GeneBank accession numbers and bootstrap values are shown within the tree.

2.3. Synthesis of dsRNA

We prepared dsRNA for RNAi experiments as previously described [30]. Briefly, the *A. pisum p300/CBP* mRNA sequence was used as a template and gene-specific RNAi primers including a 5′ T7 promoter were designed using Primer3 v4.1.0 and were purchased from Sigma-Aldrich (Germany). The dsRNA construct was designed to be 367 bp in length (GC content = 40%–60%) covering part of the ORF (Figure 1, Table S1). The construct was checked for off-targets by screening against the entire pea aphid genome, ensuring there were no overlaps >19 bp with other *A. pisum* genes. The PCR amplicon generated using the RNAi primers and cDNA template was cloned and sequenced as described above. The verified plasmid vector was used as a PCR template for the RNAi primers and the amplicon was excised from the gel and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel). The purified PCR product was used to synthesize dsRNA with the Ambion MEGAscript T7 kit (Applied Biosystems, USA). The dsRNA was purified by isopropanol precipitation and washed with ethanol. The pellet was resuspended in 30–50 µL nuclease-free water and stored −20 °C. Primers and accession numbers for all *p300/CBP* sequences used in this study are listed in Table S1.

2.4. RNAi Injection Assays

In the RNAi experiments, 5-day-old aphids were injected using glass capillaries held on a M3301 micromanipulator (World Precision Instruments, USA). The aphids were injected laterally, between the mesothorax and metathorax, with 25 nL of the *p300/CBP* dsRNA (50, 250, 1000 or 3000 ng/µL) or GFP dsRNA as a control (3000 ng/µL). We injected a total of 200 aphids per treatment, comprising five biologic replicates of 40 aphids each. After injection, aphids were individually transferred to Petri dishes containing *V. faba* leaves on 1% agarose. Aphid survival and offspring production were monitored daily as previously described [30,47]. Developmental effects were determined by tracking the start of reproduction and the number of premature (dead) offspring (Figure S1), whereas the effect on reproduction was determined by tracking the total number of viable offspring and the number of viable offspring per day. Premature nymphs were not viable after eclosion and their antennae and legs remained folded [37,48]. Newly emerged nymphs were counted daily and removed. The Petri dishes and leaves were replaced every 5 days to ensure optimal conditions. To verify the observed effects on life history traits, we additionally injected two non-overlapping *p300/CBP* dsRNA fragments (3000 ng/µL) into 40 aphids each and monitored and analyzed the above-mentioned parameters for 14 days (Table S1, Figures S2 and S3).

We also measured the body weight (0-, 3- and 8-days post-injection), size (3 and 8 days post-injection) and color (3 and 8 days post-injection) of 40 individuals treated with 3000 ng/µL p300/CBP or GFP dsRNA. To record the size and color of the aphids, images were acquired using an MZ16FA stereomicroscope (Leica Microsystems, Wetzlar, Germany) and characterized using ImageJ v1.52.

To better understand the impact of p300/CBP silencing on *A. pisum* reproduction, we dissected ovaries from aphids injected with the highest concentration (3000 ng/µL) of *p300/CBP* or GFP dsRNA 10 days post-injection. The ovaries were stored in phosphate-buffered saline with 0.1% Tween-20 (PBST) and images of the dissected specimens were acquired as described above. We counted the total number of embryos in the ovaries but also the number of late-stage embryos (stage 18 or older, defined by the presence of visible eyes) and early stage embryos (stage 17 or younger, no visible eyes) [49].

The survival of aphids was also examined in the G1 generation to evaluate possible transgenerational silencing effects. The neonate G1 nymphs (40 per treatment or control) were collected 6 days after the injection of their mothers with 3000-ng/µL *p300/CBP* or GFP dsRNA. Aphid nymphs were individually transferred to Petri dishes with *V. faba* leaves and monitored for 2 weeks.

2.5. Quantitative PCR (qPCR)

Single aphids (n = 5) were collected 12 h post-injection (3000-ng/µL *p300/CBP* or GFP dsRNA) and RNA was extracted as described above. The RNA samples were treated with TurboDNase (Invitrogen, Germany) to ensure the complete removal of genomic DNA. We then purified the RNA using the NucleoSpin RNA extraction kit. The High-Capacity RNA to cDNA kit (Applied Biosystems) was used to generate cDNA according to the manufacturer's recommendations. Gene-specific primers, designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA); http://eu.idtdna.com/PrimerQuest) and purchased from Sigma-Aldrich, were used in a 10-µL reaction to quantify the p300/CBP mRNA, comprising 10 µM specific primers, 5 µL 2x Power SYBR Green PCR Master Mix and 2 µL cDNA template (50 ng cDNA per reaction mixture). The StepOnePlus Real-Time PCR System (Applied Biosystems) was used with a primary denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. We used three replicates for statistical analysis of target gene expression with REST2009 software [50]. The data were normalized against the ribosomal protein L32 (rpl32) gene in aphids. The sequences of all primers are provided in Table S1.

2.6. Bioinformatics and Data Analysis

Protein domains were predicted using the Pfam database [51] and the NCBI conserved domains database [52]. Alignments, sequence comparisons, and the assembly of p300/CBP gene fragments were achieved using Geneious v10.2.4 (https://www.geneious.com). Multiple sequence alignment was performed using MUSCLE [53], subsequently the phylogenetic tree was built using the RAxMl plug-in [54] for Geneious v10.2.4 with default parameters. The aphid fitness data were analyzed using IBM SPSS Statistics v26 (Armonk, USA). The threshold for statistical significance was set to $p < 0.05$ for all tests, except two-way analysis of variance (ANOVA) where the threshold was $p < 0.001$. The significance of survival, evaluated by Kaplan–Meier survival analysis, and the start of reproduction, visualized as bars, were calculated using the log-rank test. The total numbers of viable and premature offspring were analyzed using the Kruskal–Wallis test with Bonferroni correction for pairwise comparisons. The number of offspring per day was analyzed by two-way ANOVA, whereas body size, body color and body weight were evaluated using Student's *t*-test.

3. Results

3.1. Genomic Sequence, mRNA Sequence and Protein Domain Analysis of A. pisum p300/CBP

The *p300/CBP* mRNA sequence from *A. pisum* clone LL01 was compared to the corresponding template sequence in the NCBI database (XM_003242184). The *A. pisum* p300-like template sequence is 7183 bp in length, whereas the size of our *p300/CBP* assembly was 6947 bp (Table S1). The assembly was produced by sequencing nine overlapping fragments of 800–1000 bp each (Table S1) and it comprises the 5'-UTR (928 bp) as well as the ORF including the ATG start codon (6019 bp). Based on the reference sequence from the NCBI (XM_008188962), the p300/CBP sequence obtained in this study is incomplete and does not include the last 134 nucleotides of the ORF, which features the stop codon and 3'-UTR. We also detected a few single nucleotide polymorphisms, but otherwise the assembly matched the *A. pisum* p300/CBP template (Figure 1A; Table S1).

Based on identified *p300/CBP* sequence in this study, a protein domain analysis revealed a distinctive set of p300/CBP domains that are conserved throughout known p300/CBP proteins of invertebrates and vertebrates. These include the KAT11 domain with acetylation activity, two TAZ-type zinc finger motifs necessary for DNA binding, a ZZ-type zinc finger with unknown function, a CBP-specific bromodomain responsible for interaction with acetylated lysine, a plant homeodomain, an atypical RING domain, as well as the characteristic KIX and CREB-binding protein-interaction domains (Figure 1B). The overall sequence is 59% identical at mRNA level and 49% identical at protein level to the *Apis melifera* p300/CBP sequences (55% at mRNA level and 42% at protein level to *Mus musculus* sequences). However, within the core catalytic region of the protein, spanning from the bromodomain to the downstream TAZ

zinc finger domain, the sequence identity to the *A. melifera* protein sequence surpasses 80%, while the rest of the sequences appeared to be less conserved. In order to confirm that the sequence identified in this study indeed represents a p300/CBP homolog, we performed a phylogenetic analysis comparing p300/CBP protein sequences from several aphid species, other insects and vertebrates (Figure 1C). The identified *A. pisum* p300/CBP sequence clustered phylogenetically together in an aphid specific subgroup, closely related to other insect species, including other hemipterans (Figure 1C).

3.2. The Effect of RNAi-Mediated Attenuation of p300/CBP on Aphid Life-History Traits

Following the injection of dsRNA, a significant decrease of *p300/CBP* transcripts was confirmed 12 h post injection using qPCR (~30% reduction; $p = 0.015$) (Figure 2).

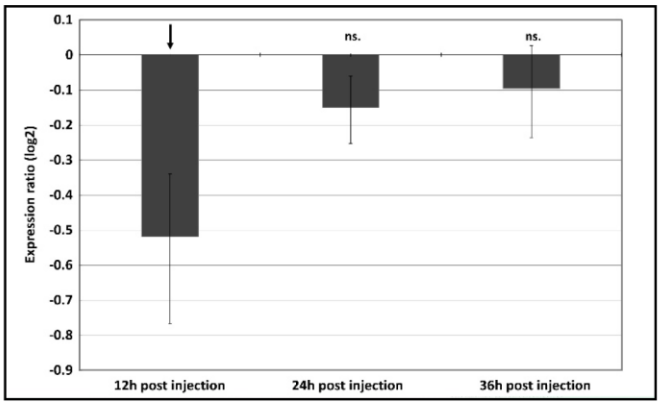

Figure 2. Expression ratio (log2) of p300 mRNA transcript determined using qPCR at 12 h, 24 h and 36 h post injection of gene-specific dsRNA in relation to the transcript expression in a GFP dsRNA treated control group. A negative expression ratio indicates downregulation, the expression was normalized against reference gene *rpl32*. Arrow indicates a significant variation of gene expression as calculated by REST analysis ($p = 0.015$). ns—not significant.

The aphid survival did not differ significantly between the *p300/CBP* dsRNA and GFP dsRNA control group during the first ~5 days post-injection. However, the overall lifespan of aphids injected with *p300/CBP* dsRNA was severely reduced (Table 1 and Table S2, Figure 3A).

Table 1. Survival frequency [%] of aphids 5-, 10-, 15- and 20-days post-injection.

Treatment	Post-Injection Survival Frequency [%]			
	After 5 Days	After 10 Days	After 15 Days	After 20 Days
GFP 3000 ng/µL	79	65	45	20
p300/CBP 3000 ng/µL	79	51	13	1
p300/CBP 1000 ng/µL	85	57	8	0
p300/CBP 250 ng/µL	88	60	11	0
p300/CBP 50 ng/µL	85	61	12	3

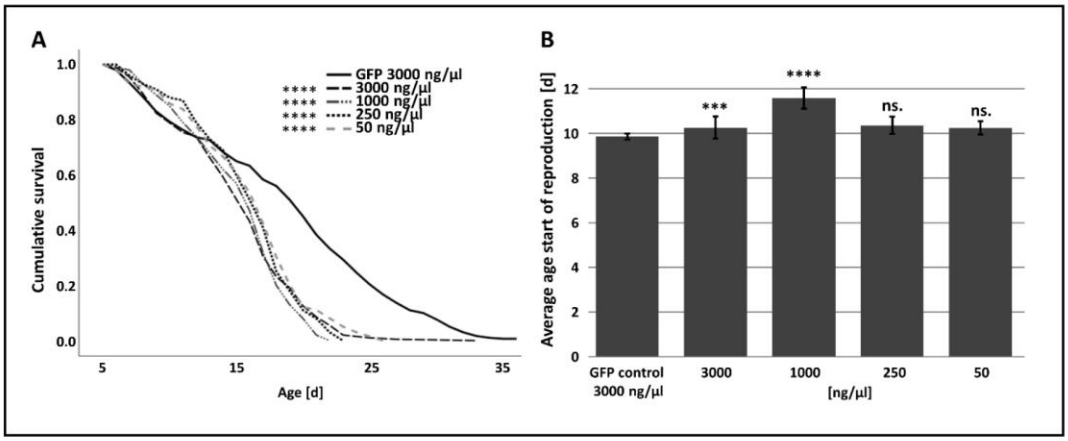

Figure 3. Life-history parameters following the injection of p300/CBP dsRNA in *A. pisum*. (**A**) Survival and (**B**) start of reproduction were monitored after the injection of 3000-, 1000-, 250- or 50-ng/µL dsRNA. Per treatment a total of 200 individuals were injected. Data were analyzed using a log-rank test. Statistical significance is indicated as follows: *** $p < 0.001$, **** $p < 0.0001$, ns—not significant.

The p300/CBP dsRNA treatment showed the strongest impact 15 days post-injection when only ~10% of aphids survived. After 20 days, there were very few survivors. In the GFP dsRNA control group, ~50% of the aphids remained alive 15 days post-injection, and 20% remained alive after 20 days (Table 1, Figure 3A).

The injection of large amounts of p300/CBP dsRNA (3000 ng/µL and 1000 ng/µL) induced a mild but significant delay to the start of the reproduction (Table S3, Figure 3B). The total number of offspring was significantly reduced in all four p300/CBP treatment groups compared to the GFP control, with 82% reduction in the 3000 ng/µL group, 82% reduction in the 1000 ng/µL group, 65% reduction in the 250 ng/µL group and 63% reduction in the 50 ng/µL group (Table S4, Figure 4A).

Further analysis revealed that the number of offspring per individual per day was also substantially lower in the *p300/CBP* dsRNA groups and was reduced in a concentration-dependent manner (Table S5, Figure 4C). Remarkably, the reproductive phase of aphids injected with *p300/CBP* dsRNA was much shorter (7–10 days) compared to the control group (up to ~25 days) (Figure 4C). The injection of p300/CBP dsRNA induced a significant increase in the number of premature nymphs throughout the reproductive phase (Tables S6 and S7, Figure 4B,D). The appearance of premature offspring indicated that p300/CBP plays a key role in aphid embryogenesis and/or eclosion.

To further verify that the observed effects are due to the suppression of p300/CBP and to minimize the possibility of off-target effects, we injected two additional, non-overlapping *p300/CBP* dsRNA constructs in the highest concentration (3000 ng/µL). The impact on life history traits of the injection of all three dsRNA fragments were comparable (Figure 3, Figure 4 and Figure S3).

The RNAi-mediated manipulation of p300/CBP did not induce changes in body weight, size or polyphenism (Tables S8 and S9, Figure 5A,B). However, the aphids injected with *p300/CBP* dsRNA became significantly darker in color 8 days post-injection, even though there were no significant differences 3 days post-injection (Table S10, Figure 5C).

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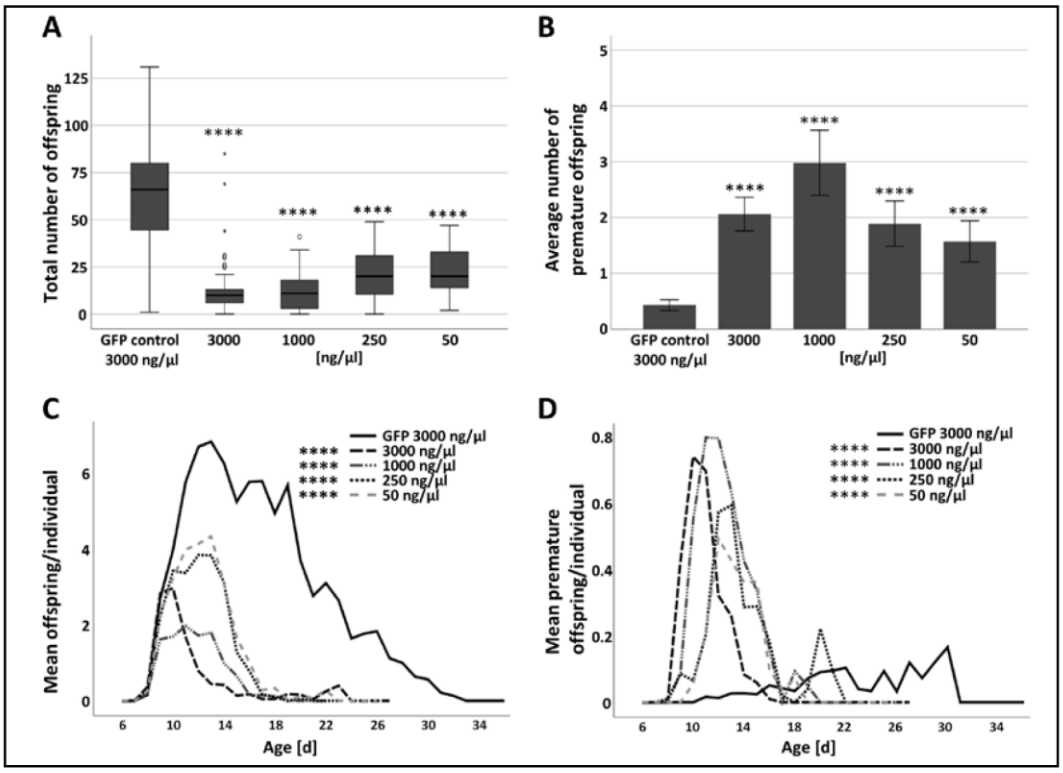

Figure 4. Reproduction parameters following the RNAi-mediated attenuation of p300/CBP in *A. pisum*. (**A**) The total number of offspring, (**B**) average number of premature offspring, (**C**) number of viable offspring per day and (**D**) number of premature offspring per day were monitored after the injection of 3000-, 1000-, 250- or 50-ng/µL *p300/CBP* dsRNA. 200 individuals per treatment were injected. To identify significant differences, we used (**A**,**B**) the Kruskal–Wallis test followed by Bonferroni corrections for pairwise analysis (**** $p < 0.0001$) or (**C**,**D**) two-way ANOVA (**** $p < 0.000001$).

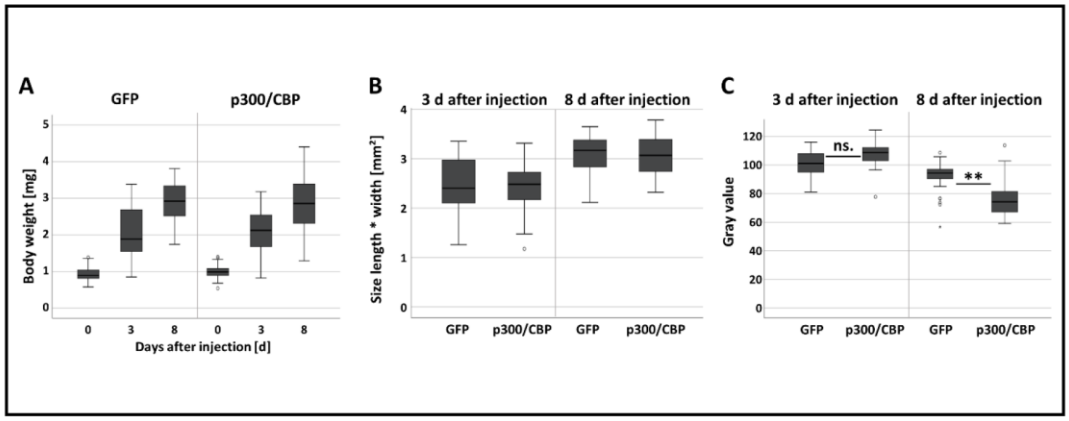

Figure 5. Life-history parameters following the RNAi-mediated attenuation of p300/CBP in *A. pisum*. (**A**) Bodyweight, (**B**) body size and (**C**) body color were determined on days 3 and 8 after treatment with 3000 ng/µL *p300/CBP* or *GFP* dsRNA. Aphid weight and size did not differ significantly between the p300/CBP treatment and GFP control groups. Data were analyzed using Student's *t*-test. Statistical significance is indicated as follows: ** $p < 0.01$, ns—not significant.

3.3. Effect of RNAi-Mediated Knockdown of p300/CBP on Aphid Embryogenesis and the Transgenerational Silencing Effect in the G1 Generation

In order to better understand the impact of p300/CBP dsRNA injection on *A. pisum* embryogenesis, we dissected ovaries from individuals in the p300/CBP treatment and GFP control groups. Ovaries dissected from aphids treated with *p300/CBP* dsRNA contained a greater number of late-stage embryos 10 days post-injection, and the tissue structure of ovaries was very fragile and highly susceptible to ruptures (Figure 6A–C). In contrast, ovaries dissected from aphids injected with the *GFP* dsRNA control had a normal tissue structure and contained embryos spanning all developmental stages (Figure 6A–C).

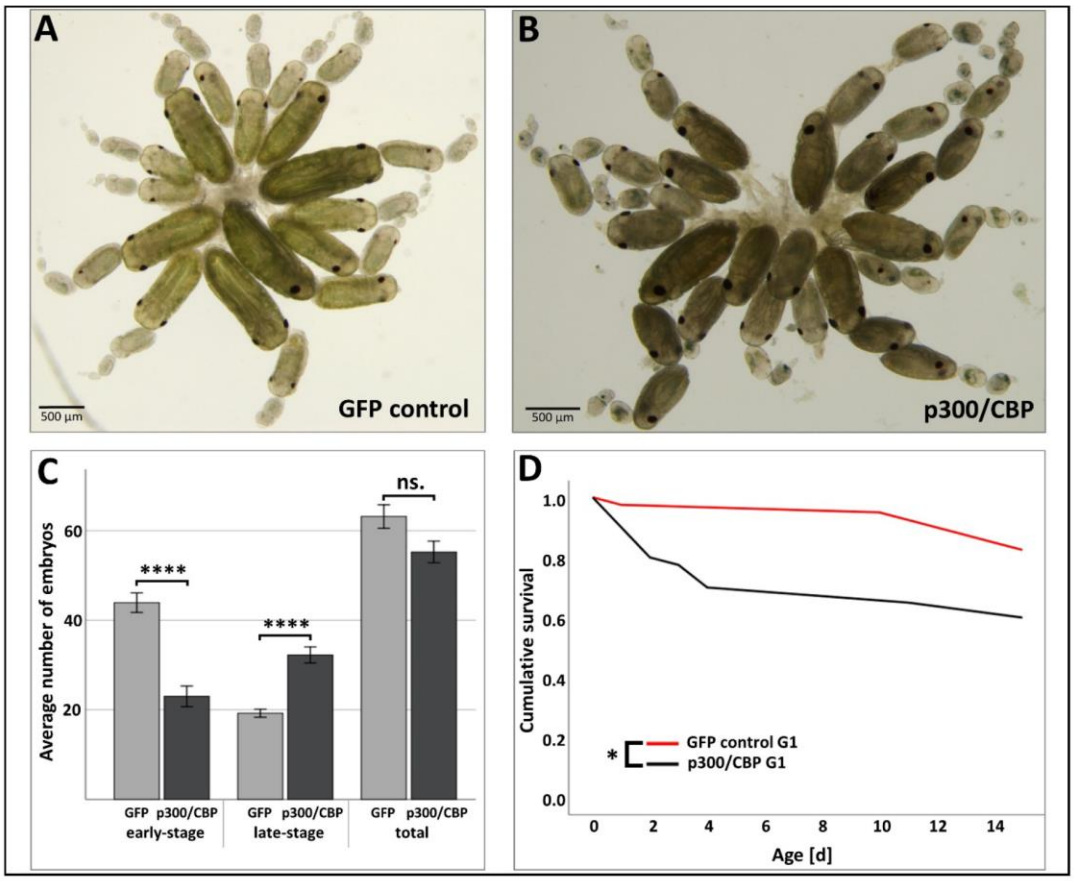

Figure 6. Effect of RNAi-mediated p300/CBP mitigation on the development and survival of the G1 generation of *A. pisum* mothers injected with dsRNA. Ovaries were dissected from aphids 10 days post-injection with (**A**) *GFP* control dsRNA or (**B**) *p300/CBP* dsRNA. (**C**) The distribution of early stage embryos (up to stage 17, no visible eyes) and late-stage embryos (stage 18 and beyond, visible eyes) differed significantly between the treatments. Ovaries from aphids treated with *p300/CBP* dsRNA contained significantly fewer early stage embryos ($p < 0.0001$) and significantly more late-stage embryos ($p < 0.0001$) than the *GFP* control group, but there was no difference in the total number of embryos. (**D**) The survival of G1 aphids from the *p300/CBP* dsRNA treatment group was compared to the GFP control group for 2 weeks. The survival of aphids in the *p300/CBP* dsRNA treatment group was significantly reduced compared to the control group ($p < 0.05$). The number of embryos was analyzed using Student's *t*-test. Survival data were evaluated using Kaplan–Meier statistics and comparisons between the treatment and control were based on log-rank tests. Statistical significance is indicated as follows: * $p < 0.05$, **** $p < 0.0001$, ns—not significant.

We monitored the survival of the viable offspring (G1 generation) of the aphids treated with *p300/CBP* dsRNA to investigate the potential for transgenerational effects. We found that the survival rate among the offspring of mothers injected with *p300/CBP* dsRNA was significantly lower than peers from the control group, whose mothers were injected with *GFP* dsRNA (Figure 6D). Although the survival of the G1 generation from the *p300/CBP* dsRNA group was affected, there were no differences in offspring count or viability between the treatment and control groups (data not shown).

4. Discussion

p300/CBP is one of the most entangled transcriptional co-regulators with hundreds of interaction partners found in a variety of multicellular organisms, from invertebrates to vertebrates [10,24,55]. In the pea aphid, p300/CBP has been identified previously in silico [31]. We further extended the characterization of aphid p300/CBP mRNA by cloning and sequencing (Figure 1 and Figure S2, Table S1). Subsequently, we were able to confirm the extensive subset of typical p300/CBP protein domains known from other species [10,55]. The core catalytic acetylation domain seems to be highly conserved and this is demonstrated by the similarities of p300/CBP in bees and aphids [10]. The phylogenetic analysis of p300/CBP protein sequences of a wide range of species, showed a clear separation of vertebrate and insect p300/CBP sequences, however, it endorsed a close relationship of all analyzed p300/CBP sequences including the one identified in our study (Figure 1C).

The analysis of insect p300/CBP protein function has focused mostly on *D. melanogaster*, but more recently the role of this protein has been investigated in *T. castaneum*, *B. germanica*, *Camponotus floridanus* and *Bombyx mori* [21,23–25,56–59]. We have expanded the scope of these experiments to include the first hemipteran model, *A. pisum*. In common with the studies involving *T. castaneum* and *B. germanica*, we used RNAi experiments to investigate the functions of p300/CBP while also evaluating its potential as a target for RNAi-mediated pest control in aphids.

The attenuation of p300/CBP in *A. pisum* resulted in severe fitness costs, supporting its role as a regulator of fundamental cellular processes as previously reported for other insects [23,24]. The injection of *p300/CBP* dsRNA significantly reduced the lifespan of the aphids as well as substantially shortening the reproductive phase, leading to the production of fewer offspring compared to peers in the *GFP* dsRNA control group (Table 1; Figures 3A and 4A,C). The inhibition of p300/CBP has previously been shown to promote senescence in human cells, prevent lifespan extension in *Caenorhabditis elegans* and increase the likelihood of apoptosis in the insect cell line BmN [60–62]. These findings are also in agreement with the low levels of p300/CBP found in aging mice [63]. A decline in longevity and fecundity is naturally correlated with biologic aging in aphids [64]. Therefore, the fitness costs observed in *A. pisum* could be an indication of senescence induced by the manipulation of p300/CBP.

The depletion of p300/CBP unexpectedly inhibited food intake in *B. germanica*, leading to the production of underdeveloped nymphs, and reduced foraging behavior in the ant *C. floridanus* [23,58]. Although the mitigation of p300/CBP in aphids delayed the start of reproduction, their body weight and body size were unaffected (Figures 3B and 5A,B). Furthermore, we observed no obvious changes in feeding behavior that would indicate a correlation between developmental costs and impaired nutrition. In *B. germanica*, p300/CBP silencing modulated the expression of genes encoding enzymes involved in gluconeogenesis and lipidogenesis [23]. Furthermore, p300/CBP dependent hyperacetylation stabilizes several nutritional storage proteins in *B. mori* [59]. It would therefore be interesting to investigate in detail whether the silencing of p300/CBP in aphids affects nutritional metabolism, but also feeding behavior.

The attenuation of p300/CBP in aphids caused an increase in the occurrence of premature (dead) offspring (Figure 4B,D), agreeing with the important role of p300/CBP during embryogenesis in *D. melanogaster*, *C. elegans* and mice [21,65,66]. This demonstrates that p300/CBP has an evolutionarily conserved function in eukaryotes. In addition to lethality, the loss of p300/CBP causes severe defects in *D. melanogaster* embryos including the absence of the head, thorax and cuticular structures [21,22]. We observed no such obvious defects in the aphid embryos produced by mothers in the *p300/CBP*

dsRNA treatment groups, but the tissue structure of ovaries was very fragile and highly susceptible to ruptures (Figure 6A,B). The lack of tissue integrity is likely to reflect the role of p300/CBP in cell-to-cell communication during organ development and morphogenesis [13]. Interestingly, obstructing p300/CBP in aphids not only increased the number of premature offspring, but also triggered the retention of embryos by their mothers (Figure 4C,D). The dissection of aphids injected with p300/CBP dsRNA revealed ovaries that contained a large number of late-stage embryos, whereas ovaries from aphids in the GFP control group contained embryos spanning all developmental stages (Figure 6A–C). Embryo retention is not a well-understood phenomenon in aphids, but it can be associated with factors ranging from disrupted embryogenesis to biologic aging [64].

The p300/CBP protein also has an important role in post-embryonic development and metamorphosis in species such as *B. germanica* and *T. castaneum* [23,24]. The knockdown of p300/CBP in *T. castaneum* suppressed the expression of more than 1300 genes encoding transcription factors and other regulatory proteins. This had numerous physiological effects, including the enhancement of melanization in the midgut as a consequence of changes in innate immunity, pigmentation and metabolism [24]. Hyperpigmentation (dark green) was observed in aphids injected with *p300/CBP* dsRNA, but this affected the whole body rather than restricted tissues (Figure 5C). Follow-up studies should investigate in detail any correlations between the disregulation of p300/CBP and components of aphid immunity such as the phenoloxidase system.

In the grain aphid (*Sitobion aveane*), RNAi-mediated silencing of the *shp* gene was shown to reduce the quantity of saliva sheath protein produced for up to seven generations [67]. Therefore, we investigated the potential transgenerational effects of p300/CBP silencing in *A. pisum*. We observed a higher mortality rate during the first few days after birth in the G1 generation of *A. pisum* from the *p300/CBP* dsRNA group, whereas aphids from the control group were unaffected (Figure 6D). We did not observe any overt morphologic aberrations in viable G1 aphids, but this does not rule out a key role for p300/CBP during post-embryonic development, as previously reported for *B. germanica* and *T. castaneum*. Future studies should investigate whether p300/CBP is involved in the post-embryonic development of aphids, perhaps through regulation of juvenile hormones and ecdysteroids, as previously shown for cockroaches and beetles [23–25].

Finally, we analyzed the expression of the endogenous *A. pisum p300/CBP* gene following the injection of *p300/CBP* dsRNA. We anticipated that *p300/CBP* dsRNA would have a direct impact on the expression of the target gene at the posttranscriptional level, but RNAi also has the potential to modify transcription by means of feedback regulation to maintain chromatin homeostasis [68]. Although the attenuation of p300/CBP caused remarkable effects on *A. pisum* life-history traits, we observed only a small change in endogenous *p300/CBP* mRNA levels (~30% reduction) 12 h post-injection (Figure 2). Given the variable efficiency of RNAi in hemipteran species, RNAi effects can be observed even if there is a low detectable impact on target gene expression [30,35,69,70]. This may be due to transient silencing that escapes detection—or may reflect the costs associated with dsRNA degradation in the hemolymph of hemipteran insects [35,71]. In addition, it has been shown in *D. melanogaster* that microRNA mediated gene silencing can occur via multiple pathways and can act through translational instead of transcriptional repression [72–74]. Hence, to verify the hypothesis of a specific dsRNA mediated dysregulation of p300/CBP and the subsequent deterioration of aphid fitness, we injected two additional non-overlapping dsRNA constructs targeting p300/CBP. The treatments resulted in the same, strong developmental as well as lifespan aberrations (Figure 3, Figure 4 and Figure S3), minimizing the chances that the observed phenotypic alterations are off-target effects. Besides the moderate reduction of p300/CBP transcripts it is possible that protein levels were lower in aphids due to the inhibition of translation, which would require the quantitation of p300/CBP by western blot or similar methods [75,76]. Follow up studies need to clarify the importance of translational repression of dsRNA mediated gene silencing in aphids, which could also add another layer of complexity to the anyway challenging RNAi mediated gene repression in aphids.

5. Conclusions

In conclusion, we have shown that the RNAi-mediated inhibition of p300/CBP has a remarkably potent negative impact on life-history traits in *A. pisum*, significantly contributing to our understanding of p300/CBP as a universal transcriptional co-regulator in insects [24]. It would be valuable to investigate the function of p300/CBP in more detail by RNAi-mediated silencing followed by differential gene expression analysis to identify p300/CBP target genes in *A. pisum* as previously shown in beetles [24]. The RNAi-mediated control of aphids and other pest insects has already been demonstrated by the development of transgenic crops expressing dsRNA [38,39,45,67,77]. The transgenic approach may not be efficient for every pest and every crop, therefore growing evidence supports the utilization of dsRNA spray formulations as next-generation insecticides. The targeting of p300/CBP in this manner could provide an efficient and environmentally sustainable approach to reduce the agricultural damage caused by aphid pests.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/5/265/s1, Figure S1: Nymphs of p399/CBP dsRNA treated mothers, Figure S2: The *A. pisum p300/CBP* mRNA sequence from clone LL01 with RNAi target sides, Figure S3: Life history parameters following the RNAi-mediated silencing of p300/CBP, Table S1: Primer sequences used in this study, Table S2: Statistical analysis of RNAi data for survival compared to the GFP control, Table S3: Statistical analysis of RNAi data for start of reproduction compared to the GFP control, Table S4: Statistical analysis of RNAi data for average number of offspring compared to the GFP control, Table S5: Reproductive parameters evaluated during the RNAi experiments including viviparous offspring determined by two-way ANOVA, Table S6: Statistical analysis of RNAi data for average number of premature offspring compared to the GFP control, Table S7: Reproductive parameters evaluated during the RNAi experiments including premature offspring determined by two-way ANOVA, Table S8: Statistical analysis of RNAi data for body weight [mg] compared to the GFP control (dsRNA concentration = 3000 ng/µL), Table S9: Statistical analysis of RNAi data for body size (length × width)(mm^2) compared to the GFP control (dsRNA concentration = 3000 ng/µL), Table S10: Statistical analysis of RNAi data for body color (grayscale) compared to the GFP control (dsRNA concentration = 3000 ng/µL), Sequence of p300, Supplementary Fragments: *A. pisum* p300/CBP mRNA, with primer positions underlined and indicated in bold (see Table S1).

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5.3 3RD PUBLICATION – CO-AUTHOR PUBLICATION

3 Fitness costs of infection with *Serratia symbiotica* are associated with greater susceptibility to insecticides in the pea aphid *Acyrthosiphon pisum*

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Declaration of contribution:

In this paper I contributed in conceiving and designing the experimental setup. I took part in the execution of the experiments and data collection. Additionally, I was responsible for the data analysis and supported in data interpretation.

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Fitness costs of infection with *Serratia symbiotica* are associated with greater susceptibility to insecticides in the pea aphid *Acyrthosiphon pisum*

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Abstract

BACKGROUND: Aphids are agricultural pests that damage crops by direct feeding and by vectoring important plant viruses. Bacterial symbionts can influence aphid biology, e.g. by providing essential nutrients or facilitating adaptations to biotic and abiotic stress.

RESULTS: We investigated the pea aphid (*Acyrthosiphon pisum* Harris) and its commonly associated secondary bacterial symbiont *Serratia symbiotica* to study the effect of this symbiont on host fitness and susceptibility to the insecticides imidacloprid, chlorpyrifos methyl, methomyl, cyantraniliprole and spirotetramat. There is emerging evidence that members of the genus *Serratia* can degrade and/or detoxify diverse insecticides. Therefore, we hypothesized that *S. symbiotica* may promote resistance to these artificial stress agents in aphids. Our results showed that *Serratia*-infected aphids were more susceptible to most of the tested insecticides than non-infected aphids. This probably reflects the severe fitness costs associated with *S. symbiotica*, which negatively affects development, reproduction and body weight.

CONCLUSION: Our study demonstrates that *S. symbiotica* plays an important role in the ability of aphid hosts to tolerate insecticides. These results provide insight into the potential changes in tolerance to insecticides in the field because there is a continuous and dynamic process of symbiont acquisition and loss that may directly affect host biology.

Supporting information may be found in the online version of this article.

Keywords: bacterial symbionts; Hemiptera; host fitness; insecticide tolerance; pest

1 INTRODUCTION

Aphids are agricultural pests that feed on phloem sap, causing the stunting, discoloration and deformation of plants, while the growth of sooty molds on honeydew produced by these insects reduces the economic value of crops.[1] These hemipterans are also vectors of many important plant viruses.[2] The control of aphids relies predominantly on insecticides such as carbamates, organophosphates, pyrethroids, neonicotinoids and pymetrozine.[3] The frequent use of insecticides over decades has led to multiple forms of aphid resistance to most classes of insecticides, making some species of aphids (e.g. the green peach aphid *Myzus persicae* Sulzer) very difficult to control.[3–5]

Aphids live in intimate association with bacterial symbionts that can influence their biology.[6,7] This group of insects rely on the primary symbiont *Buchnera aphidicola*, a vertically transmitted bacterium that supplements the nutrient-deficient diet with essential amino acids.[8] In addition, aphids may carry one or more bacterial symbionts that are not strictly necessary for host survival and reproduction, but can influence their adaptation under specific environmental conditions.[6] These bacteria are known as secondary symbionts, and their precise roles in their insect hosts are not fully understood. Furthermore, secondary symbionts can be found in different locations within the aphid body, frequently colocalized with *B. aphidicola* in specialized host cells known as bacteriocytes.[9,10] Although the secondary symbionts of insects are maternally transmitted with high fidelity, they are occasionally transferred horizontally, allowing them to spread within and between species.[11] Potential transmission routes include shared host plants or parasitoids, resulting in the direct acquisition of novel ecological traits.[9,11–14]

Secondary symbionts can act as mutualists (e.g. providing protection against stress or natural enemies) or parasites (e.g. manipulating reproduction) in the host.[7] The expressed phenotype of

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a secondary symbiont depends on the environmental conditions, but also sometimes on the symbiont strain.[15,16] Some symbionts of aphids are known to provide their hosts with protection against parasitoids and heat stress, particularly *Serratia symbiotica*, one of the most common secondary symbionts of aphids, and *Hamiltonella defensa*, which has been investigated comprehensively in the context of host–symbiont interactions.[11,17–21] Despite the known protection they confer, maintaining symbionts, especially in the absence of stress, can be also very costly for the aphid host. For example, the black bean aphid (*Aphis fabae* Scopoli) infected with *H. defensa* is strongly protected against parasitoids, but in the absence of these natural enemies the host has a shorter lifespan and a lower rate of reproduction.[22] The level of protection and associated fitness costs to the host can sometimes depend on the symbiont strain.[23] For example, when *A. fabae* is not exposed to parasitoids, strongly protective strains of *H. defensa* are less costly for the host than weaker strains in terms of lifespan and reproduction.[19] This shows the complexity of interactions between aphids, their symbionts and environmental conditions.

There is emerging evidence that bacteria (including symbionts) are involved in the detoxification or degradation of insecticides, which may help to address pollution issues but on the other side could also promote insecticide resistance.[24–28] *Serratia*, *Burkholderia*, *Pseudomonas* and *Flavobacterium* spp. can degrade several different classes of insecticides.[27,29–33] *Serratia* spp. may carry plasmids encoding enzymes such as hydrolases, which correlate with the degradation of insecticides.[33] Such enzymes have also been found in *Burkholderia* spp., which are associated with insecticide resistance in the stink bug (*Riptortus pedestris* Fabricius).[28,34]

However, the presence of some symbiotic bacteria is known to increase host susceptibility to insecticides.[35–37] This is mainly associated with the physiological costs of infection and the consequential reduced fitness of the host. For example, Asian citrus psyllid (*Diaphorina citri* Kuwayama) infected with *Candidatus* Liberibacter are more susceptible to several classes of insecticides.[35] The presence of *Wolbachia* spp. in mosquitoes (*Culex pipiens* L.) does not directly affect host susceptibility to insecticides, but the presence of insecticide-resistance genes in *C. pipiens* negatively affects its life history traits and therefore ability to control density of this symbiont.[38,39]

Despite the given examples of symbionts involved in host tolerance to chemical insecticides, this field remains largely uninvestigated. In this study, we hypothesized that *S. symbiotica* might facilitate *A. pisum* in the detoxification of insecticides, because members of *Serratia* spp. can produce hydrolases that degrade such compounds. We also investigated the impact of *S. symbiotica* on *A. pisum* fitness, correlating this with the role the symbiont plays in determining the susceptibility of aphids to insecticides commonly used in agriculture.

2 MATERIALS AND METHODS

2.1 Maintenance of aphids and establishment of the *Serratia*-free line

A. pisum parthenogenetic clone LL01 was maintained on the host plant *Vicia faba* var. *minor* as previously described.[40,41] Age-synchronized aphids were used in all experiments and were kept on detached, mature *V. faba* leaves on agar under controlled conditions.[40,42]

A *Serratia*-free aphid line was established using antibiotics as previously described,[43] with minor modifications. *A. pisum* G0 nymphs (1 day old) were maintained in feeding chambers and fed for 3 days on an artificial AP3 diet[44] containing 500 µg/mL ampicillin.[45] The aphids were then transferred to agar plates containing *V. faba* leaves and reared until they produced G1 offspring.[46] Afterwards, 50 G1 individuals (1 day old) were treated as above and reared to adulthood. Their G2 offspring were used to start the *Serratia*-free line, after the absence of the symbiont was confirmed by polymerase chain reaction (PCR) in the G1 mothers. The infection status of the *Serratia*-free line was periodically confirmed by PCR. Hereafter, the original aphid line is described as *Serratia*-positive. The cured aphid line was strictly separated from *Serratia*-positive line to prevent contamination. To eliminate any potential side-effects of the treatment, the *Serratia*-free line was continuously reared on *V. faba* plants for at least 10 generations before it was used for experiments.

2.2 Detection of symbionts in *A. pisum*

Bacterial symbionts in *A. pisum* were detected by extracting total DNA from the insect samples listed in Table 1 using the CTAB method as previously described.[47] Aphid saliva was collected[48] and DNA was extracted as above for further symbiont detection. For honeydew collection, a *V. faba* plant infested with aphids was placed 10 cm above a Petri dish for 2 h, and the collected honeydew droplets were washed with a small amount of double-distilled water before DNA was extracted as above.

A 1.5-kb segment representing bacterial 16S rRNA genes was amplified from pooled adult individuals, tissues or secretions (Table 1) by PCR using the universal primers 16SA1 and 16SB1 (Table S1).[49] The reaction volume was 25 µL, comprising 4 µL DNA template (25 ng/µL), 10 µM of each primer (1 µL), 12.5 µL of GoTaq Green Master Mix (2×) (Promega, Mannheim, Germany) and 6.5 µL of nuclease-free water. PCR products were visualized by 1% agarose gel electrophoresis using SYBR Safe (Invitrogen, Germany), and eluted using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany).

The PCR products were transferred to vector pGEM-T Easy (Promega) and introduced into RapidTrans TAM1 competent *Escherichia coli* cells (Active Motif, Carlsbad, CA, USA). The transformed cells were spread on MacConkey agar (Carl Roth, Karlsruhe, Germany) and incubated at 37 °C overnight. The colonies were then screened for the PCR insert using the standard T7 primer set. Positive colonies were cultivated overnight in 5 mL lysogeny broth containing 100 µg/mL ampicillin in a shaking incubator at 200 rpm and 37 °C. Plasmid DNA from randomly chosen and independent colonies (Table 1) was purified using the NucleoSpin Plasmid EasyPure kit (Macherey-Nagel) and sequenced for verification on a 3730xl DNA analyzer (Macrogen Europe, Amsterdam, The Netherlands). The sequences were compared against the NCBI databases using BLAST.[50]

2.3 Insecticides and aphid bioassays

We used insecticides from different chemical classes that are currently used for aphid control (http://www.irac-online.org/documents/sucking-pests-moa-poster/).[3] Most of the tested compounds act on insect nerve and muscle targets: imidacloprid (neonicotinoid)-acts on the nicotinic acetylcholine receptor (nAChR) in the central nervous system causing hyperexcitation; chlorpyrifos methyl (organophosphate) and methomyl (carbamate)-inhibit acetylcholinesterase (AChE) causing hyperexcitation, and cyantraniliprole (diamide)-activates muscle ryanodine receptors, causing contraction and paralysis. Spirotetramat (tetramic acid derivative) inhibits acetyl coenzyme A

Table 1. Detection of *Serratia symbiotica* in *Acyrthosiphon pisum* (clone LL01) tissues and secretions

Sample*	No. of bacterial 16S rRNA gene clones	Most commonly detected bacteria†	*S. symbiotica* GenBank accession number
Whole body (adults)	20		
Salivary glands	48		
Gut	48	*S. symbiotica*	MF062646-MF062653
Saliva	20		
Honeydew	20		

	Presence (+)/absence (−)†	
	S. symbiotica	*B. aphidicola*
Whole body (adults)	+	+
Salivary glands	+	-
Gut	+	-
Saliva	+	-
Honeydew	+	-

*Pool of insect specimens or tissues ($n = 10$) and aphid secretions (saliva and honeydew) in at least three biological replicates were analyzed by DNA extraction, diagnostic PCR and sequencing.
†Bacterial taxon with the number of sequenced clones. The taxon was designated according to the bacterial genus of the BLAST hit with >96% sequence identity.

carboxylase, which is involved in the synthesis of lipids responsible for insect growth and development (Table 2). All insecticides were purchased from Chem Service Inc. (West Chester, PA, USA). *Serratia* spp. have mainly been tested for their ability to degrade synthetic organic insecticides, which have a long history of application and many associated issues with toxicity and environmental pollution.[33,51,52]

For each insecticide, a stock solution of 1000 µg/mL was prepared in acetone and then working solutions were diluted with distilled water. To determine LC_{50} values, we tested six concentrations (100, 25, 6.25, 1.56, 0.39 and 0.0975 µg/mL) of each insecticide.

Aphid bioassays were performed as suggested by the Insecticide Resistance Action Committee (http://www.irac-online.org/methods/aphids-adultnymphs/) with minor modifications. The effect of the insecticides was scored on nymphs (3 days old) of the *Serratia*-positive and *Serratia*-free aphid lines. The assay was performed by dipping *V. faba* stems with roots into plastic vials containing the compound for 24 h. Afterwards, Petri dishes with treated leaf discs on agar were prepared as above. Ten nymphs were transferred to each leaf disc in six replicates for each concentration. Each experiment was conducted with three biological replicates. The mortality of aphids was scored daily over 3 days of exposure. The corresponding solvent and water controls were used during each experiment.

2.4 Fitness measurements

Fifty aphid nymphs (0–1 days old), from the two aphid lines were randomly selected and individually reared on Petri dishes containing *V. faba* leaves on agar as above. Fresh leaves were provided every 5 days to maintain the aphids in good conditions. *A. pisum* mothers from the *Serratia*-positive and *Serratia*-free lines and their randomly collected offspring were tested by PCR for the presence of *S. symbiotica*.

To determine the effect of *S. symbiotica* on the aphids, several parameters were monitored until death. Aphid survival and offspring production were monitored daily, and newly emerged nymphs were counted and removed. In addition, we recorded the appearance of wings, premature nymphs and molts. Body weight and the color of maternal aphids was scored when they were 12 days old. Body color was recoded under a Leica MZ 16 FA stereomicroscope as previously described.[53] The effect on reproduction was determined by recording the start of reproduction, total number of offspring, number of offspring per day and number of premature nymphs. Developmental effects were determined by recording the day of the last molt before reproduction and the day of the last molt.

2.5 Data analysis

We analyzed the data using IBM SPSS Statistics v23 software (Armonk, New York, NY, USA). Statistical significance was defined

Table 2. Susceptibility of *Serratia*-positive and *Serratia*-free aphid lines to five chemical insecticides

Primary site of action and main group of insecticide (IRAC, 2017)		Active ingredient	LC_{50} (µg/mL) *Serratia*-positive	LC_{50} (µg/mL) *Serratia*-free
Nerve action	Nicotinic acetylcholine receptor (nAChR) competitive modulators	Imidacloprid	0.005	0.006
	Acetylcholinesterase (AChE) inhibitors	Chlorpyrifos methyl	>100	>100
		Methomyl	10.96	25.12
Nerve and muscle action	Ryanodine receptor modulator	Cyantraniliprole	0.069	0.158
Lipid synthesis, growth	Inhibitors of acetyl CoA carboxylase	Spirotetramat	0.240	5.248

Table 3. Summary of fitness parameters measured for the two aphid lines in this study

Fitness parameters		Aphid line* *Serratia*-positive	Aphid line* *Serratia*-free	Significance
Body weight (mg)		2.57 ± 0.80	3.53 ± 0.52	$P < 0.0001$
Total number of offspring		84.82 ± 4.53	109.04 ± 2.97	$P < 0.001$
Start of reproduction (d)		9.40 ± 0.09	8.51 ± 0.07	$P < 0.0001$
Last molt to reproduction (d)		1.74 ± 0.11	1.20 ± 0.05	$P < 0.01$
Day of the last molt (d)		7.58 ± 0.12	7.31 ± 0.07	$P < 0.05$
		Statistical parameters†		
No. of offspring per day	Aphid line (*Serratia*-positive, *Serratia*-negative)	df = 1, mean square = 35.57, $F = 7.94$		$P < 0.05$
	Day of reproduction	df = 35, mean square = 539.01, $F = 120.35$		$P < 0.0001$
	Aphid line × day of reproduction	df = 35, mean square = 102.52, $F = 22.89$		$P < 0.0001$

*Mean ± SE.
†Determined by two-way ANOVA.

as $P < 0.05$ for all the tests in this study, except the two-way analysis of variance (ANOVA) where the threshold was $P < 0.001$. For fitness measurements, survival data representing the two aphid lines were analyzed by Kaplan–Meier survival analysis and comparisons between the groups were based on log-rank tests. The number of offspring per day was analyzed by two-way ANOVA, whereas body weight and body color were analyzed using Student's *t*-test. All the other fitness parameters listed in Table 3 were analyzed using the Mann–Whitney U test.

For the insecticide bioassays, the total mortality for each insecticide treatment was corrected according to Abbott's formula based on mortality scored in the control groups.[54] Mortality in the control groups ranged between 0 and 17%. The results were analyzed using non-linear sigmoid curve fitting, and the activity of each insecticide was tested based on the dose response concentrations (LC_{50}). We used Student's *t*-test to compare mortality between the two aphid lines.

3 RESULTS

3.1 *A. pisum* infection with bacterial symbionts

Initial screening of bacterial symbionts in a laboratory population of *A. pisum* revealed infection solely with *B. aphidicola* and *S. symbiotica*. Both symbionts were detected in all individuals, but *S. symbiotica* was not detected in the *Serratia*-free line, confirming the successful elimination of this symbiont (data not shown).

In addition, *S. symbiotica* was detected in aphid tissues (gut and salivary glands) and its secretions (honeydew and saliva) (Table 1).

3.2 Susceptibility of *Serratia*-positive and *Serratia*-free *A. pisum* lines to insecticides

The *Serratia*-positive and *Serratia*-free aphid lines were exposed to five commonly used insecticides, namely imidacloprid, chlorpyrifos methyl, methomyl, cyantraniliprole and spirotetramat (Table 2). Each insecticide was tested at three concentrations (high, medium and low) although the absolute concentration in each case was specific for each compound, based on the LC_{50} value (Fig. 1, Table 2). As expected, high mortality was observed at the highest concentration of each compound regardless of the presence or absence of *S. symbiotica*. The efficacy of the insecticides ranged from high (imidacloprid, cyantraniliprole and spirotetramat) to extremely low (chlorpyrifos methyl). In some cases, the insecticides showed different LC_{50} values in the two aphid lines (Table 2). We found that the *Serratia*-positive line was significantly more sensitive to low concentrations of imidacloprid (0.0975 µg/mL), methomyl (1.56 µg/mL), chlorpyrifos methyl (6.25 µg/mL) and to both medium and low concentrations of spirotetramat (1.56 and 0.39 µg/mL) than the *Serratia*-free line (Fig. 1). We observed no differences in mortality when the two aphid lines were treated with the three different concentrations of cyantraniliprole.

3.3 Effect of *S. symbiotica* on *A. pisum* life history traits

Before conducting fitness experiments, we rescreened both aphid lines by diagnostic PCR to confirm the presence or absence of the symbiont. We observed severe developmental and reproduction costs in aphids infected with *S. symbiotica* (Fig. 2, Table 3). Development of the aphids was monitored by tracking molting parameters, whereas the impact on reproduction was monitored by measuring reproductive delay and by counting the number of offspring. We observed a significant delay in the development of aphids infected with *S. symbiotica* compared with the non-infected aphids (Fig. 2b,c). Furthermore, infected aphids reproduced later than their uninfected peers and produced fewer offspring in total and on a per-day basis (Fig. 2d–f). In addition, the non-infected aphids were heavier than the infected aphids, perhaps reflecting the greater abundance of embryos within the females, eventually leading to the production of more offspring (Fig. 2e,f, Table 3). Other parameters, such as survival, body color and the frequency of premature nymphs, did not differ significantly between the two aphid lines (Figs S1–S3, Table S2).

4 DISCUSSION

Many groups of pest insects live in symbiosis with bacteria that may provide them with fitness benefits under specific ecological conditions, but there may also be severe costs that raise the question of how these symbiotic relationships are maintained over time.[7,55,56] The role these symbionts play in host resistance to chemical insecticides has received less attention, although they can be a source of metabolic innovations for their host insects.[25,28,57,58] There is growing evidence to support the involvement of insect-associated bacterial symbionts in detoxification of chemical insecticides, e.g. the stink bug *R. pedestri* and its symbiont *Burkholderia* spp., the diamondback moth (*Plutella xylostella*

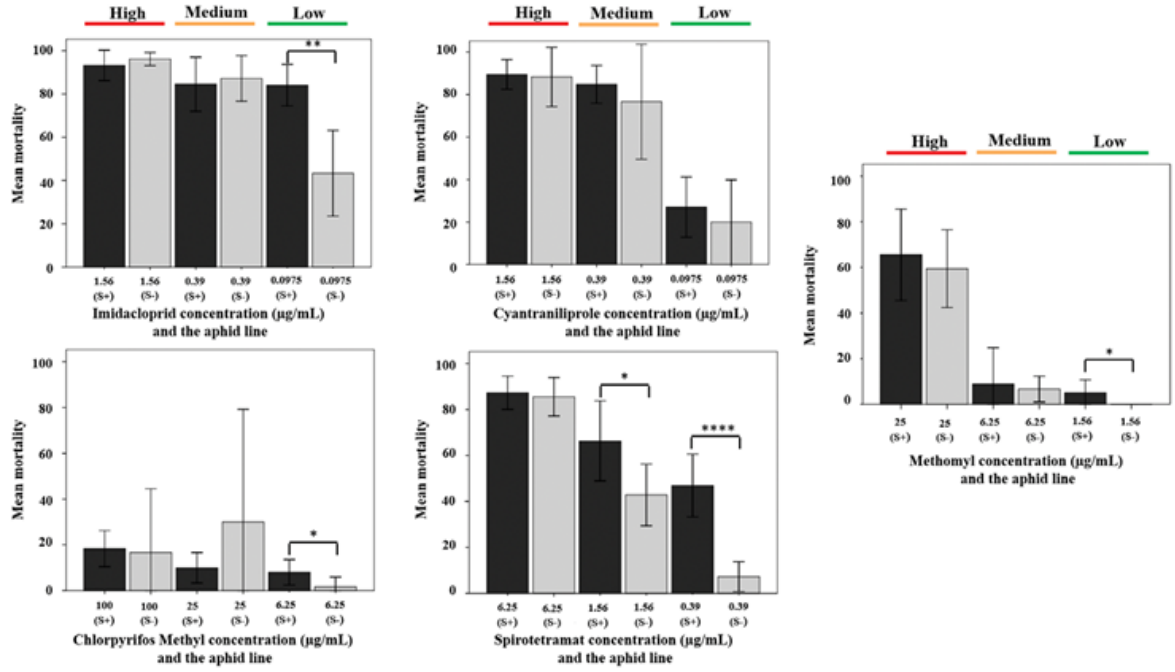

Figure 1. Mortality of *Serratia*-positive (S(+), dark-gray box plot) and *Serratia*-free (S(−), light gray box plot) aphid lines following exposure to insecticides (Table 2). Each insecticide was tested at three concentrations (high, medium and low) depending on LC_{50} values (Table 2). Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

L.) and the symbionts *Bacillus*, *Enterobacter* and *Pantoea* spp., and the fruit fly (*Bactrocera dorsalis* Hendel) with associated *Citrobacter* spp.[25,26,59] These bacteria are mainly associated with the detoxification of organophosphates, although *Bacillus* spp. can also detoxify oxadiazine insecticides.[26,28,60,61]

S. symbiotica is one of the most common symbionts of aphids with predominantly mutualistic functions.[11,21,23,62] Some members of genus *Serratia* are known to be involved in the degradation or detoxification of insecticides and plant toxins.[25,30–32] We hypothesized that *S. symbiotica* may help its aphid hosts to better tolerate exposure to chemical insecticides. Surprisingly, our results contradicted this hypothesis and instead revealed that our *Serratia*-positive aphid line was significantly more susceptible to exposed insecticides than a non-infected line (Fig. 1, Table 2).

Previous studies have investigated the correlation between the presence of bacterial symbionts and the susceptibility of another phloem sap-sucking pest, *B. tabaci*, to chemical insecticides.[36,37] In agreement with our data, *B. tabaci* lines carrying the symbiont *Rickettsia* were more susceptible to insecticides from different chemical groups (acetamiprid, thisamethoxam, spiromesifen and pyriproxifen).[37] Furthermore, *B. tabaci* became more susceptible to insecticides, including imidacloprid, when simultaneously infected with *Rickettsia* and *Arsenophonus* or *Wolbachia* and *Arsenophonus*.[36] In addition, *P. xylostella* infected with gut symbiont *Serratia* spp. was more susceptible to the insecticide chlorpyrifos.[63] We have shown here and in our previous work that *S. symbiotica* can invade various tissues of the pea aphid, as is also the case for *Rickettsia* spp. in *B. tabaci*, which increased the sensitivity of the host to insecticides (Table 1).[41,64–66]

Insect hosts need to keep their symbiotic community under control to ensure the success of both partners.[55,67] This frequently involves a trade-off between the control of symbionts and investment in development, survival and reproduction.[55] Previous studies have shown that symbionts like *H. defensa*, *S. symbiotica*, *Regiella insecticola*, *Rickettsia* spp. and *Spiroplasma* spp. can negatively affect aphid survival and reproduction depending on the strain of host and symbiont, and also on presence or absence of stress conditions.[20,55,68,69]

In this study, we assumed that the higher sensitivity of the *Serratia*-positive aphid line to most of the insecticides we tested would also be associated with the abovementioned trade-off. Accordingly, fitness experiments revealed clear differences in life history parameters between *Serratia*-positive and *Serratia*-free aphid lines (Fig. 2, Table 3). There were no differences between the lines in terms of survival, but the *Serratia*-positive line had a much smaller average body weight and both development and reproduction were negatively affected.

As previously reported, *S. symbiotica* benefits its aphid host during heat stress and parasitoid attack.[11,21,55] However, depending on the strain of *S. symbiotica* and specific environmental conditions there may be associated costs that could increase host susceptibility to chemical insecticides, as shown here. Our fitness data are in broad agreement with Laughton *et al.*,[55] who showed that *S. symbiotica* confers higher costs in terms of pea aphid survival, development and fecundity compared with other bacterial symbionts (*H. defensa* and *R. insecticola*). These fitness parameters could be also improved if pea aphids infected with *S. symbiotica* are exposed to certain stress factors, such as heat.[23]

In their natural environments, insects experience a continuous process of symbiont acquisition and loss, with host plants often serving as a source of symbiont acquisition, as shown for *Rickettsia* spp. in *B. tabaci*.[13,70] In our study, detection of *S. symbiotica* in the salivary glands and saliva of *Serratia*-positive aphids gives a hint

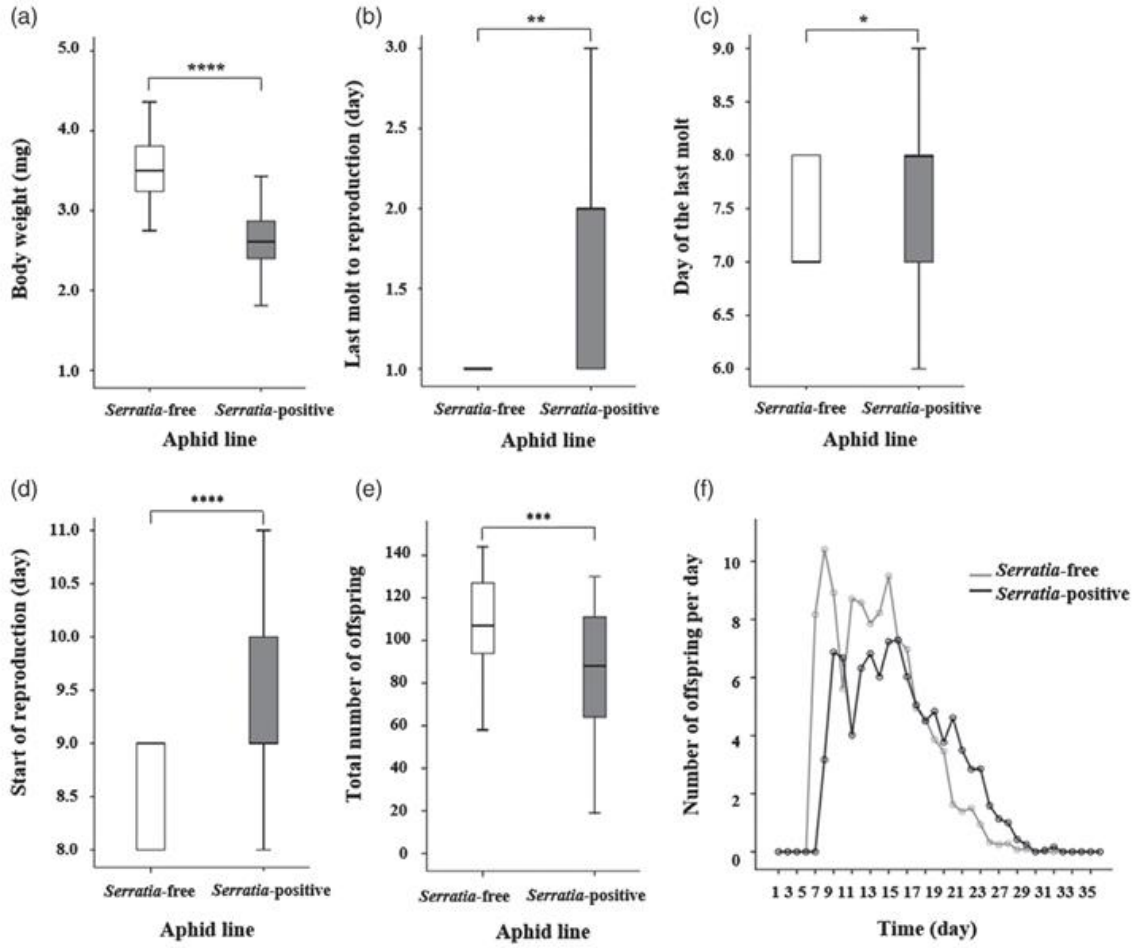

Figure 2. Effects of *Serratia symbiotica* on *Acyrthosiphon pisum* life history parameters: number of molts (a), last molt to reproduction (b), day of last molt (c), start of reproduction (d), total number of offspring (e), number of offspring per day (f) and body weight (g). *Serratia*-free (white box plot) and *Serratia*-positive (gray box plot) aphid lines were evaluated to determine the effect of the symbiont on host fitness. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

that this symbiont in addition to vertical transmission, may also use some of the potential horizontal routes for transmission (e.g. via the plant) (Table 1). In general, these transmission events can result in the instantaneous acquisition of ecologically important traits, which could also involve resistance or susceptibility to insecticides.[6,12,71] Himler *et al.*[72] observed the rapid spread of *Rickettsia* spp. among *B. tabaci* in the southwestern USA. These *Rickettsia*-infected *B. tabaci* had higher survival rates, faster development and more female offspring. At the same time, it is likely that these whiteflies could have higher susceptibility to chemical insecticides, as shown in Israeli populations.[37] Surprisingly, Ghosh *et al.*[73] reported a trend in the abundance of symbiont-free *B. tabaci* populations in South Africa with improved fitness parameters, but also plant virus vectoring abilities. Such examples of the sudden spread or a loss of a symbiont in insect populations may influence host sensitivity to insecticides and could be a key determinant of the efficacy of insecticide applications will be in the field.

As mentioned above, remarkable variations between specific environmental conditions and the roles symbionts play within their hosts may reflect not only the presence of different symbionts, but also different strains of a particular symbiont species.[17] Some strains may confer a range of non-protective through to highly protective phenotypes in the host, and the level of protection attracts costs as previously shown in the example of *A. fabae* and *H. defense*.[11,19] Among the aphids, the greatest strain diversity has been reported for *H. defensa*, although other symbionts such as *Arsenophonus* spp., *S. symbiotica*, *Rickettsia* spp., *Wolbachia* spp. and *Spiroplasma* spp. also exist in different strains.[17,74–76]

Follow-up studies should test other aphid-associated symbionts and their strains to investigate in more detail the correlation between the bacterial community and host tolerance to chemical insecticides. Further research should also be carried out to determine the benefits conferred upon aphids that maintain symbionts as costly as *S. symbiotica*.

Our study found that fitness costs of common bacterial symbiont of aphids significantly influence their ability to tolerate insecticides. This suggests that some symbionts of aphids could be key players in determining changes in tolerance to these artificial stress agents in the field.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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5.4 4TH PUBLICATION – CO-AUTHOR PUBLICATION

4 Orally delivered scorpion antimicrobial peptides exhibit activity against pea aphid (*Acyrthosiphon pisum*) and its bacterial symbionts.

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Phillipp Kirfel, & Andreas Vilcinskas,

Toxins (2017), 9 (9)

Declaration of contribution:

My contribution to this paper was performing the experiments. I was taking microscopy pictures and collecting survival data during the feeding experiements.

toxins

MDPI

Article

Orally Delivered Scorpion Antimicrobial Peptides Exhibit Activity against Pea Aphid (*Acyrthosiphon pisum*) and Its Bacterial Symbionts

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Abstract: Aphids are severe agricultural pests that damage crops by feeding on phloem sap and vectoring plant pathogens. Chemical insecticides provide an important aphid control strategy, but alternative and sustainable control measures are required to avoid rapidly emerging resistance, environmental contamination, and the risk to humans and beneficial organisms. Aphids are dependent on bacterial symbionts, which enable them to survive on phloem sap lacking essential nutrients, as well as conferring environmental stress tolerance and resistance to parasites. The evolution of aphids has been accompanied by the loss of many immunity-related genes, such as those encoding antibacterial peptides, which are prevalent in other insects, probably because any harm to the bacterial symbionts would inevitably affect the aphids themselves. This suggests that antimicrobial peptides (AMPs) could replace or at least complement conventional insecticides for aphid control. We fed the pea aphids (*Acyrthosiphon pisum*) with AMPs from the venom glands of scorpions. The AMPs reduced aphid survival, delayed their reproduction, displayed in vitro activity against aphid bacterial symbionts, and reduced the number of symbionts in vivo. Remarkably, we found that some of the scorpion AMPs compromised the aphid bacteriome, a specialized organ that harbours bacterial symbionts. Our data suggest that scorpion AMPs holds the potential to be developed as bio-insecticides, and are promising candidates for the engineering of aphid-resistant crops.

Keywords: *Acyrthosiphon pisum*; scorpion toxins; symbiosis; antimicrobial peptides

1. Introduction

Aphids are among the most destructive agricultural pests, causing direct damage to crops by feeding on phloem, as well as indirect losses by transmitting viruses [1]. Aphids are also biological models for the investigation of insect–plant interactions and symbiosis [2]. *Buchnera aphidicola* is an obligate bacterial symbiont of aphids, and is exclusively localized in a specialized structure known as bacteriome, which consists of bacteriocytes. This species has coevolved with aphids to provide them with essential amino acids that are not supplied in sufficient quantities by the sugar-rich phloem sap on which aphids feed [3,4]. Aphids also frequently host one or more secondary bacterial symbionts,

including *Serratia symbiotica*, *Hamiltonella defensa*, and *Regiella insecticola* [4]. These symbionts colonize different aphid tissues and provide several functions, including protection against natural enemies, heat stress tolerance, a supply of nutrients, and adaptation to the host plant [5–7].

The pea aphid *Acyrthosiphon pisum* (Harris) was selected among more than 4700 known aphid species for the first aphid genome sequencing project [8]. The most surprising revelation was that *A. pisum* has a greatly reduced repertoire of innate immunity genes when compared to other insects [9–11]. The *A. pisum* genome lacks genes encoding classical antimicrobial peptides (AMPs), and also lacks components of the immune deficiency pathway [10]. However, *A. pisum* is not completely defenceless against pathogens because it has several genes encoding thaumatins, which confer antifungal activity in other insects [9]. Recently, genes encoding short peptides resembling AMPs were identified in the aphid bacteriome, and these may be used to control bacterial symbionts [12]. Nevertheless, the limited innate immune system in *A. pisum* is likely to represent a protective adaptation that helps to maintain long-lasting symbiosis with bacteria [10,11,13,14]. Accordingly, AMPs may provide the basis for alternative insecticides that can be expressed in crops, given that any harm to symbionts would inevitably affect the aphids themselves [15–17]. This strategy relies on the ability of orally ingested AMPs to function correctly, even when exposed to peptidases and proteases found in the aphid gut [18].

Scorpions are predatory arachnids that feed on small arthropods (mainly insects). Their venom components have evolved over more than 450 million years into specialized toxins that efficiently kill their prey [19]. Their venom contains a cocktail of bioactive compounds, including neurotoxins that target mammals and/or insects, and amongst others, AMPs. Scorpion AMPs belong to the non-disulfide-bridged peptides (NDBP) family, which have diverse biological functions, including antimicrobial, bradykinin-potentiating, and immunomodulatory activities [20–23]. Scorpion AMPs have only been investigated against human pathogenic bacteria showing low minimal inhibitory concentrations (MICs), even against multi-drug-resistant bacteria [20,21]. However, their use as antibiotics has proven challenging due to their mildly haemolytic activity [19].

Given that scorpions prey on insects, we attempted to broaden the use of scorpion AMPs by investigating their insecticidal activity against *A. pisum*. Several AMPs recently identified in the venom gland transcriptome of the scorpion *Urodacus yaschenkoi* (Birula) (UyCT1, UyCT3, UyCT5, Uy17, Uy192 and Uy234) have been produced as synthetic peptides and tested in vitro against human pathogenic bacteria [24–26]. Another AMP (Um4) was identified in the venom of the black rock scorpion *Urodacus manicatus* (Thorell) [27]. The antimicrobial activities of these naturally occurring scorpion AMPs were compared to modified analogues (designed peptides, herein named D-peptides) generated by exchanging some amino acids and inserting positively charged residues to increase the net positive charge of the AMPs, and hence their affinity for bacterial membranes. None of the natural or engineered scorpion AMPs were active against fungi, but many were active at low MICs (0.25–30 μM) against seven different bacteria [28].

Here, we selected scorpion AMPs with low MICs and low haemolytic activity in order to test their activity against aphids and aphid bacterial symbionts both in vitro and in vivo. Their activities were compared to three insect-derived AMPs, as well as the antibiotic rifampicin and the insecticide imidacloprid. Our data suggest that scorpion-derived AMPs are promising candidates for the development of bio-insecticides and aphid-resistant transgenic plants.

2. Results

2.1. Effect of AMP Treatments on Aphid Survival

The effect of each AMP was determined by tracking aphid survival during three days of feeding (Figure 1, Table 1 and Table S1). The insect AMPs did not affect aphid survival, whereas the scorpion AMPs were highly effective in killing the aphids. Some of the scorpion AMP treatments (UyCT3, UyCT5, and D3) were highly effective at all tested concentrations, whereas others (Uy17, Uy192,

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Uy234, D5, D10, and D11) were effective only at the medium (250 μg/mL) and high (500 μg/mL) concentrations, and UyCT1 and Um4 were only effective at the highest concentration.

We used the insecticide imidacloprid and the antibiotic rifampicin as controls to gauge the effectiveness of AMP treatments, although their modes of action differ from AMPs. Imidacloprid killed all the aphids in less than three days (survival rate 0%), whereas rifampicin did not significantly affect aphid survival as compared to the control AP3 diet (survival rate 92.5%).

Table 1. Effect of antimicrobial peptides (AMPs) and control treatments after three days of feeding.

Treatment		Concentration (μg/mL)	% Survival	Significance ^
Insecticide	Imidacloprid	5	0	****
Antibiotic	Rifampicin	50	92.5	ns
Scorpion AMPs	UyCT1	50	96	ns
		250	76	ns
		500	28.6	****
	UyCT3	50	70	*
		250	52.5	****
		500	35	****
	UyCT5	50	68	**
		250	27.5	****
		500	10	****
	Uy17	50	75	ns
		250	4	****
		500	0	****
	Uy192	50	82	ns
		250	57.5	****
		500	12	****
	Uy234	50	85	ns
		250	0	****
		500	20	****
	Um4	50	85	ns
		250	90	ns
		500	55	****
	D3	50	72.5	*
		250	72.5	*
		500	32.5	****
	D5	50	83.7	ns
		250	42.5	****
		500	22	****
	D10	50	86	ns
		250	0	****
		500	0	****
	D11	50	86.7	ns
		250	57.1	**
		500	64	**
Insect AMPs	Apidaecin	50	100	ns
		250	90	
		500	83.3	
	Cecropin A	50	80	
		250	83.3	
		500	85	
	Stomoxyn	50	68	
		250	68	
		500	75	

^ Compared to control AP3 diet (survival = 90%); ns—not significant, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

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Survival curves were constructed to compare the insecticidal activity of scorpion and insect AMPs in *A. pisum* (Figure 1, Table S1). Figure 1A shows the effect of the insect AMPs (500 μg/mL) compared with the control AP3 diet, imidacloprid (5 μg/mL) and scorpion AMP UyCT5 (500 μg/mL). The three insect AMPs (cecropin A, apidaecin and stomoxyn) had no significant effect (survival rate ≥75%) against *A. pisum* whereas UyCT5 was highly effective at the same concentration (survival rate <10%). Figure 1B compares three UyCT AMPs, which reduced aphid survival by 65–90%. UyCT5 was the most effective, killing 10% of the nymphs after the first day and more on the second and third days until only ~10% of the aphids survived. Figure 1C compares the *U. yaschenkoi* AMPs Uy17, Uy192, Uy234 and the *U. manicatus* AMP Um4, revealing that Uy17 was the most potent. Figure 1D compares the D-peptides, indicating that D10 was the most effective, killing all aphids by the end of the third day.

These data show that some of the scorpion AMPs are comparable to imidacloprid in terms of potency, e.g., Uy17 and Uy234 at the highest concentration, and D10 at the medium and highest concentrations, resulting in 100% mortality (Figure 1). Aphids that survived the three days of treatment were monitored for the following two weeks in order to detect any delayed effects of the AMP treatments. In most cases, there was no significant difference in survival as compared to the control AP3 diet. However, the survival rate continued to decline in the aphid groups fed on 50 μg/mL Um4, 50 and 500 μg/mL Uy234, and 500 μg/mL D5 (data not shown).

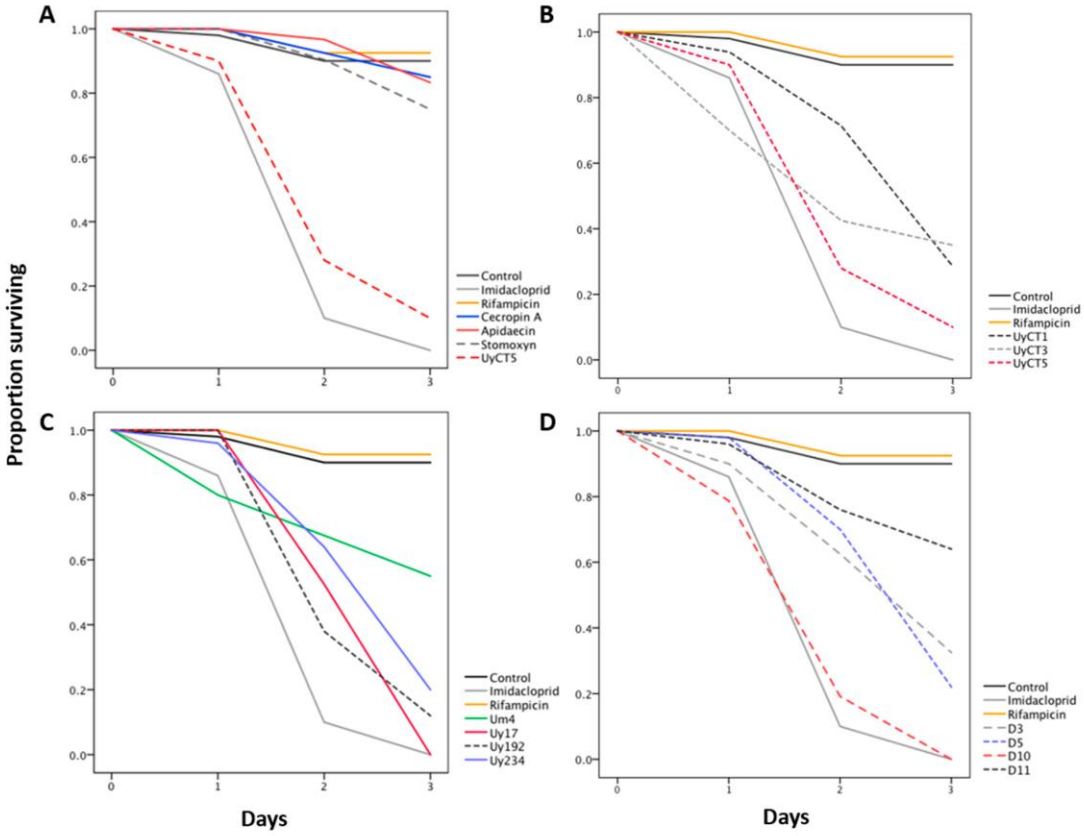

Figure 1. Insecticidal activity of scorpion and insect AMPs in *A. pisum*. Aphid survival was monitored during three days of feeding on an AP3 diet mixed with the corresponding AMP. Survival data were evaluated by Kaplan-Meier analysis. Statistical data are shown in Table S1. The insecticide imidacloprid was used as a positive control (5 μg/mL). (**A**) Insect AMPs, 500 μg/mL. (**B**,**C**) Natural scorpion AMPs, 500 μg/mL. (**D**) Designed scorpion AMPs (D-peptides), 500 μg/mL. The most effective AMPs were UyCT5, Uy17, Uy192, and D10, causing ~90% mortality. Insect AMPs had no significant effect on aphid survival.

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2.2. *Effect of AMP Treatments on Aphid Reproduction*

The effect of each AMP on *A. pisum* reproduction was determined by counting the number of offspring and recording the delay before reproduction in surviving aphids for two weeks after treatment (Figure 2).

Most of the scorpion and insect AMP treatments affected the time to reproduction, in some cases even at the lowest tested concentration of 50 μg/mL, resulting in significant delays of several days as compared to the control AP3 diet (Figure 2A). The treatments that did not cause a significant reproductive delay were 500 μg/mL UyCT1, 500 μg/mL UyCT5, 500 μg/mL Uy192, 50 μg/mL Uy234, 500 μg/mL D3, and 500 μg/mL D5.

Most of the treatments did not affect the number of offspring regardless of the effect on survival (Figure 2B). However, all three concentrations of cecropin A, the 500 μg/mL apidaecin treatment, and the 500 μg/mL Uy234 treatment had a significant impact on the number of offspring.

Rifampicin caused the most significant effect on reproduction, resulting in smaller and underdeveloped adults that produced hardly any offspring (Figure 2B). However, rifampicin did not affect aphid survival after three days of feeding nor during the two weeks after treatment (data not shown).

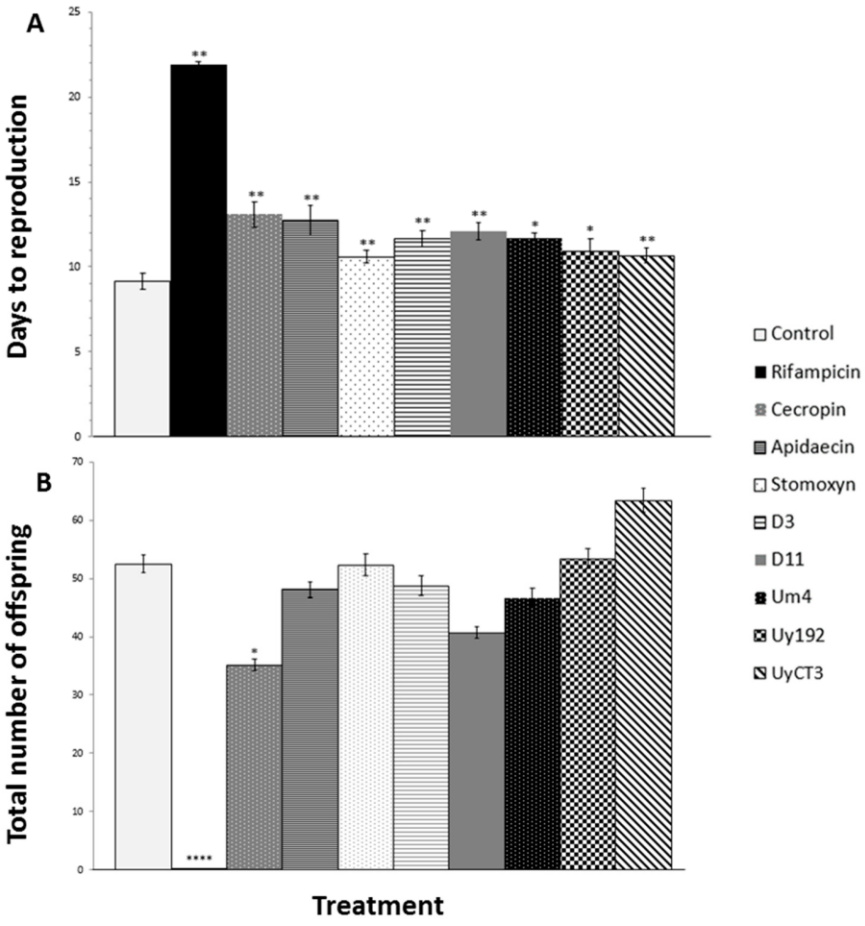

Figure 2. Impact of representative AMPs and antibiotic treatments on the time to reproduction and the number of offspring in *A. pisum*. (**A**) Days to reproduction increased after the treatments. (**B**) The number of offspring was not significantly affected by most of the treatments, except rifampicin and cecropin A. Negative control = control (AP3 diet); positive control = 50 μg/mL rifampicin; AMP treatments = 250 μg/mL. Statistical significance: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.3. Effect of AMPs on Bacterial Growth In Vitro

The susceptibility of aphid symbionts to AMPs was tested using the only known cultivable strain for aphids: *S. symbiotica* CWBI-2.3 [29]. Most of the scorpion AMPs that affected aphids in the feeding experiments also showed in vitro activity against *S. symbiotica* CWBI-2.3, with MICs of 125–500 μg/mL corresponding to the in vivo range (Table 2). Interestingly, some scorpion AMPs that were active in the feeding assays showed no in vitro activity against *S. symbiotica* CWBI-2.3, even at the highest tested concentration of 500 μg/mL (Uy234, Um4, D10 and D11) suggesting that they affect the aphids without targeting these bacterial symbionts. Further research is required to determine the mode of action of such compounds. Insect AMPs were not active against *S. symbiotica* CWBI-2.3, even at 500 μg/mL.

Table 2. Efficacy of treatments used against the bacterial symbiont *S. symbiotica* CWBI-2.3.

Compounds		MIC (In Vitro) (μg/mL)
Scorpion AMPs	UyCT1	125
	UyCT3	125
	UyCT5	125
	Uy17	250
	Uy192	500
	Uy234	>500
	Um4	>500
	D3	250
	D5	500
	D10	>500
	D11	>500
Insect AMPs	Apidaecin	>500
	Cecropin A	>500
	Stomoxyn	>500
Antibiotics	Rifampicin	50

MIC—minimal inhibitory concentration.

2.4. qPCR-Based Quantification of Bacterial Symbionts in Treated Aphids

We used a quantitative PCR (qPCR) assay to determine the impact of scorpion AMPs on the population density of *S. symbiotica* and *B. aphidicola* in vivo. Two groups of samples were analysed: (i) after three days of AMP treatments in feeding chambers, and (ii) two weeks after treatment. These samples were compared to investigate whether *S. symbiotica* and *B. aphidicola* can recover from AMP exposure. As shown in Figure 3, many scorpion AMPs significantly reduced the density of *S. symbiotica* and *B. aphidicola* after three days as compared to the control AP3 diet: 50, and 250 μg/mL D3, 50, and 500 μg/mL D11, all three tested concentrations of UyCT3, and UyCT5, 250 μg/mL Uy192, and 500 μg/mL Um4. Furthermore, the density of symbionts was still significantly lower than the control level after two weeks in the groups treated with 50 μg/mL D3, 250, and 500 μg/mL D11, 250, and 500 μg/mL Um4, and all three concentrations of UyCT3 and UyCT5 (data not shown).

Rifampicin caused a significant reduction in the numbers of *S. symbiotica* and *B. aphidicola* after three days of exposure and two weeks after treatment, but none of the insect AMPs reduced the density of either symbiont. Indeed, both symbionts were slightly more abundant two weeks after the treatment with cecropin A.

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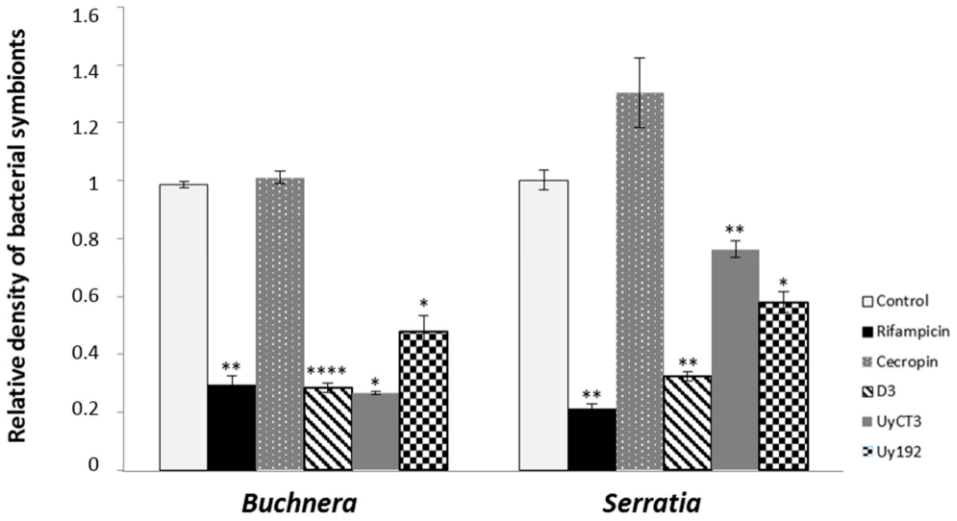

Figure 3. Quantitative PCR for the detection of *Buchnera aphidicola* and *Serratia symbiotica* in the *A. pisum* after AMP and antibiotic treatments. Data show the relative abundance of symbionts after three days of exposure for representative treatments (left panel = *B. aphidicola*; right panel = *S. symbiotica*). Negative control = control AP3 diet; positive control = 50 µg/mL rifampicin; AMP treatments = 250 µg/mL. Statistical significance indicated as follows: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.5. Localization of Bacterial Symbionts in A. pisum by Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was carried out with specific probes (Table S2) to establish the tissue distribution of *S. symbiotica* and *B. aphidicola* in aphids 24 and 48 h after exposure to the highest concentration of each AMP and the control treatments. In the negative control (AP3 diet) group, we found that *B. aphidicola* was exclusively localized in bacteriome of the nymphs, and its associated ovarioles (Figure 4A,C), whereas *S. symbiotica* was detected in most tissues, including the gut, bacteriome, and ovarioles (Figure 4B,C). The *S. symbiotica* signal remained visible in aphid tissues 24 h after the AMP treatments, and was prevalent in the gut (Figure 4D). However, the signal could not be detected after 48 h, indicating that the *S. symbiotica* 16*S* rRNA had degraded by this point (Figure 4E). We also found that treating aphids with the scorpion AMPs compromised the structure of the bacteriome (Figure 4E). Rifampicin treatment also reduced the *S. symbiotica* signal after 48 h, whereas the signal remained strong 48 h after treatment with the three insect AMPs (data not shown).

In contrast to the results observed for *S. symbiotica*, neither the AMPs nor rifampicin reduced the intensity of the signal for *B. aphidicola* in aphid nymphs. However, the *B. aphidicola* signal was often detected in the siphunculi 48 h after treatment with D3, D5, D10, Um4, UyCT3, and UyCT5 (Figure 4E), which might indicate that the bacteriome structure has been compromised. As expected, imidacloprid did not affect the localization of the bacterial symbionts, because it acts directly on the insect central nervous system.

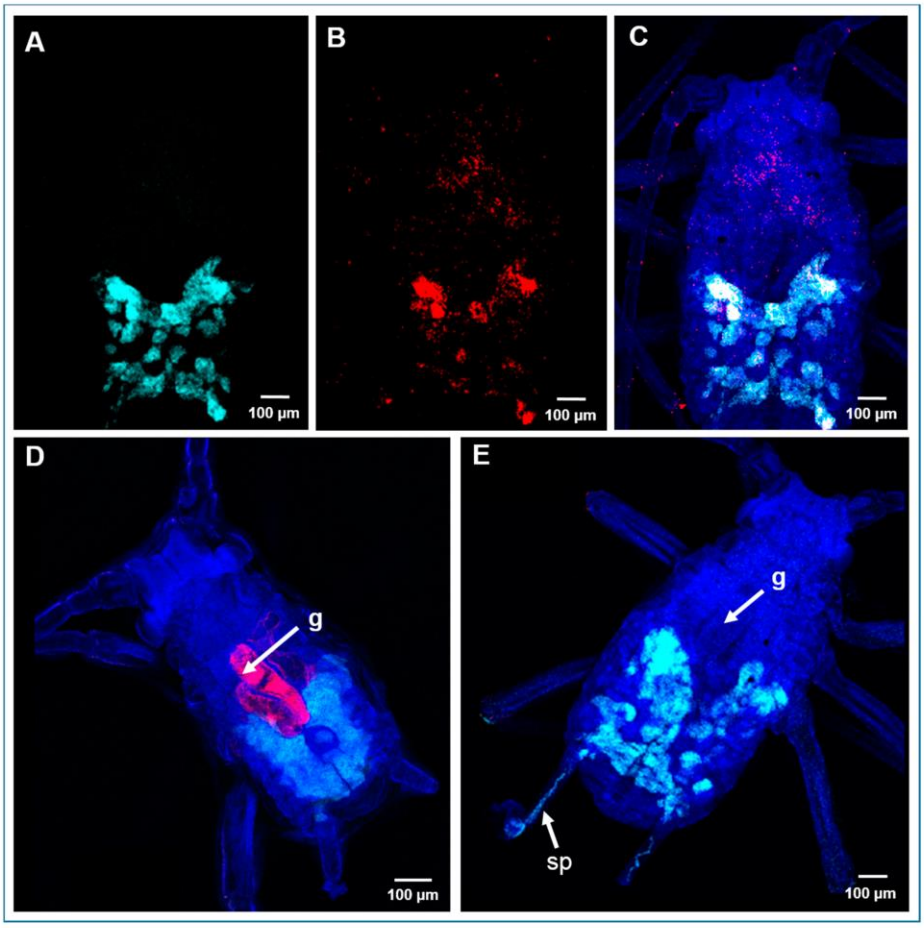

Figure 4. Localization of bacterial symbionts by fluorescence in situ hybridization (FISH) in *A. pisum* nymphs before and after treatment with scorpion AMPs. Specific probes were used for *Buchnera aphidicola* (light blue) and *Serratia symbiotica* (red). (**A**) Detection of *B. aphidicola*. (**B**) Detection of *S. symbiotica*. (**C**) Double FISH for the detection of both symbionts in untreated (control AP3 diet) *A. pisum* nymphs. (**D**) *S. symbiotica* and *B. aphidicola* in aphid nymphs after exposure to scorpion AMPs (e.g., UyCT3) for 24 h, and (**E**) 48 h. DAPI (dark blue) was used as a nuclear counterstain. Abbreviations: g = gut; sp = siphunculi.

3. Discussion

Aphids are dependent on their association with bacterial symbionts, and antibiotics can therefore impair their fitness and fecundity [16,30]. The evolution of innate immunity in aphids has been accompanied by the loss of many genes encoding antibacterial peptides because their expression could damage bacterial symbionts [14]. This has led to a hypothesis in which engineered crops expressing AMPs could be used to target aphids via their bacterial symbionts [31,32]. Engineered pathogen-resistant crops already provide a sustainable strategy to counteract specific plant diseases. For example, the antifungal peptides gallerimycin from *Galleria mellonella* (Linnaeus) and metchnikowin from *Drosophila melanogaster* (Meigen) have been shown to confer fungal resistance in plants [33,34].

As previously stated, the efficacy of AMPs expressed in crops relies on the ability of orally ingested AMPs to function correctly even following exposure to digestive enzymes found in the aphid gut [18]. We therefore investigated whether feeding aphids with scorpion and insect AMPs can affect their survival and fecundity. We selected AMPs from two Australian scorpion species (*U. yaschenkoi* and *U. manicatus*) because the evolution of scorpions has involved the development of venom glands

producing peptides and proteins that can efficiently kill insect prey [24,26]. Certain scorpion AMPs are also active against human pathogenic bacteria [21,28]. We used three insect AMPs (cecropin A, apidaecin and stomoxyn), as well as a synthetic insecticide (imidacloprid) and antibiotic (rifampicin) as controls to evaluate the scorpion AMPs.

Each of the scorpion AMPs we tested was active against *A. pisum*, affecting their survival and/or fecundity. UyCT3, UyCT5, and D3 were highly effective at all three tested concentrations, whereas UyCT1 and Um4 were effective only at the highest concentration (500 μg/mL). In contrast, the insect AMPs we tested had no effect on aphid survival, and only a minimal impact on reproduction (Figure 2). In addition, many of the tested scorpion and insect AMPs delayed reproduction, but only a few reduced the number of offspring (cecropin A, apidaecin and Uy234) (Figure 2). The impact of scorpion and insect AMPs on aphid reproduction is probably a non-specific consequence of AMP toxicity, which causes an overall decrease in the fitness of aphids, and thus impairs their reproductive ability.

One potential explanation for the differential activity of scorpion and insect AMPs against aphids and their bacterial symbionts is the origin and intrinsic characteristics of these peptides. Scorpion AMPs are short cationic amphipathic peptides that are produced in the venom gland [23]. They target cell membrane by a pore-forming mechanism resulting in the loss of electrolytes [35]. Their broad activity against bacteria, erythrocytes, and other mammalian cells has been attributed to their lack of selectivity. Their precise function in nature still remains unclear, but they may protect the telson (open end of the fifth metasomal segment) from bacterial infections and may also help neurotoxins reach their targets once the AMP has ruptured the cell membrane [19,36].

The insect AMPs used in this study are expressed in the haemolymph when the host insect is challenged by a pathogen [37–40]. These AMPs act selectively against the membranes of a wide range of human, animal, and plant bacterial pathogens, but they do not affect eukaryotic cells [41–43]. Insect AMPs usually disrupt bacterial membranes by forming pores, but the mechanism of apidaecin is different [35]. This proline-rich AMP not only breaches the bacterial membrane, but also binds intracellular targets. The ineffectiveness of insect AMPs in aphids may reflect their selective nature toward pathogens, whereas scorpion AMPs target different tissues, including the bacteriome, probably using the same lytic mode of action.

As well as assessing the impact of each AMP on aphid survival and fecundity, we evaluated their direct effect against both *B. aphidicola* and *S. symbiotica*. The CWBI-2.3 strain of *S. symbiotica* is the only aphid symbiont that can be cultivated under laboratory conditions [29]. This strain is a transitional form between a free-living bacterium and a host-dependent mutualistic symbiont, and is a close relative of the *S. symbiotica* strain found in the *A. pisum* population used in this study [44]. We were able to determine MICs for each AMP against *S. symbiotica* CWBI-2.3 in vitro. Most of the scorpion AMPs (UyCT1, UyCT3, UyCT5, Uy17, Uy192, D3, and D5) inhibited the growth of *S. symbiotica* CWBI-2.3 (Table 2). We found that several of the scorpion AMPs that affected aphid performance in the feeding assays were also active against *S. symbiotica* in vitro and in vivo, whereas others (Uy234, Um4, D10, and D11) did not act directly against the symbiont but were nevertheless active against the aphids in feeding assays, suggesting an alternative mechanism of action or an alternative target.

We also investigated the effect of the AMPs by using qPCR and FISH to directly characterize the population density and localization of both *B. aphidicola* and *S. symbiotica* in aphid tissues. FISH analysis did not reveal any clear AMP-mediated effect on the abundance of intracellular *B. aphidicola*, but there was a remarkable reduction in the *S. symbiotica* population, which was more accessible to the AMPs due to its intracellular and extracellular localization (Figure 4D,E). However, qPCR revealed a significant reduction in the density of both populations, confirming the antibacterial effect of the tested scorpion AMPs (Figure 3).

The compartmentalization of symbionts inside the bacteriome and specialized host-derived membranes is an evolutionary strategy to protect mutualistic symbionts from host innate immunity, including AMPs [45]. This special structure must be breached before AMPs can exert their antibacterial activity [46–49]. For these reasons, the selective insect AMPs were probably unable to reach the

bacterial symbionts, whereas the non-selective scorpion AMPs were more likely to compromise the bacteriome, affecting both symbionts (Figures 3 and 4E).

The observed insecticidal and antibacterial activities of scorpion AMPs against *A. pisum* and its bacterial symbionts are supported by earlier research in which indolicidin, an AMP from bovine neutrophils, showed activity against the green peach aphid *Myzus persicae* (Sulzer) and also affected the bacteriome [50]. Furthermore, scorpion AMPs (UyCT3, UyCT5, Uy192, Um4, D11) and indolicin showed activity against *Escherichia coli*, which is closely related to *B. aphidicola*, providing further support for our observations [20,21,28,51,52].

In summary, we found that the scorpion AMPs UyCT3, UyCT5, and D3 were the most effective against aphids and their symbionts. These AMPs showed insecticidal activity at different concentrations and they clearly affected aphid survival and reproduction, but also significantly reduced the population size of both *B. aphidicola* and *S. symbiotica*. There is a growing interest in the development of bio-insecticides derived from the venom of arachnids that prey on insects [53–56]. The natural characteristics of scorpion AMPs make them attractive candidates for this purpose because they are short and linear, and therefore easy to synthesize at low costs. Scorpion AMPs are also suitable candidates for the engineering of aphid-resistant crops, although further research is required to determine whether there are any negative effects in the plants themselves and whether the scorpion AMPs confer a significant degree of protection against aphids when expressed *in planta*.

4. Materials and Methods

4.1. Antimicrobial Peptides

We used the natural scorpion AMPs UyCT1, UyCT3, UyCT5, Uy17, Uy192, and Uy234 from *U. yaschenkoi*, and Um4 from *U. manicatus* [24,26,27]. Enhanced UyCT peptides (herein named D-peptides) were modified to increase membrane affinity [28]. The UyCT group of peptides was synthesized by Biomatik Corporation (Cambridge, ON, Canada) at 98% purity. The remaining scorpion peptides were synthesized by Caslo ApS (Lyngby, Denmark) at 98% purity. All peptides were amidated at the C-terminus. The scorpion AMPs selected for this study were chosen based on their activity against human pathogenic bacteria [21,28], and those with low MICs and haemolytic values were preferred (Table 3). Three insect AMPs were tested as controls: cecropin A from the moth *Hyalophora cecropia* (Linnaeus), apidaecin from the bumblebee *Bombus pascuorum* (Scopoli), and stomoxyn from the stable fly *Stomoxys calcitrans* (Linnaeus) [37,39,40]. These insect AMPs display antibacterial activity against a broad range of Gram-negative and Gram-positive bacteria [42]. Insect AMPs were synthetized by Coring System Diagnostix (Gernsheim, Germany) at >90% purity.

Table 3. List of AMPs and control compounds tested against *A. pisum* and its bacterial symbionts.

Compounds		Sequence or Chemical Formula
Scorpion AMPs	UyCT1	GFWGKLWEGVKNAI
	UyCT3	ILSAIWSGIKSLF
	UyCT5	IWSAIWSGIKGLL
	Uy17	ILSAIWSGIKGLL
	Uy192	FLSTIWNGIKGLL
	Uy234	FPFLLSLIPSAISAIKRL
	Um4	FFSALLSGIKSLF
	D3	LWGKLWEGVKSLI
	D5	GFWGKLLEGVKKAI
	D10	FPFLKLSLKIPKSAIKSAIKRL
	D11	GFWGKLWEGVKNAIKKK
Insect AMPs	Apidaecin	GNRPVYIPPPRPPHPRL
	Cecropin A	KWKL FKKIEKVGQN IRDGIIKAGPAVAVVGQATQIA
	Stomoxyn	RGFRKH FNKLVKKVKH TISETAHVAKDTAVIAGSGA AVVAAT
Antibiotic	Rifampicin	$C_{43}H_{58}N_4O_{12}$ (CAS number 13292-46-1)
Insecticide	Imidacloprid	$C_9H_{10}ClN_5O_2$ (CAS number 138261-41-3)

4.2. Aphids and the Detection of Bacterial Symbionts

A. pisum clone LL01 was reared on 2–3-week-old bean plants (*Vicia faba* var. minor) in a climate cabinet (KBWF 720, Binder GmbH, Tuttlingen, Germany) with a 16-h photoperiod and a day/night temperature of 24/18 °C, as previously described by [57]. Plants for experiments and aphid rearing were cultivated in a greenhouse at an average temperature of 20 °C under natural light, plus additional illumination (SONT Agro 400 W, Phillips, Eindhoven, The Netherlands) to maintain a 14-h photoperiod.

The *A. pisum* population was screened for the presence of bacterial symbionts, as previously described [58,59], with slight modifications. Total genomic DNA was isolated from individual aphids or pools of 10–20 aphids using the CTAB method [60]. Bacterial symbionts were detected by PCR using genus-specific primers to amplify 16*S* rRNA gene fragments (Table S2) [59,61]. The reaction volume was 25 µL, comprising of 4 µL DNA template (25 ng/µL), 10 µM of each primer (1 µL), 12.5 µL of GoTaq Green 2x Master Mix (Promega, Madison, WI, USA) and 6.5 µL nuclease-free water. PCR products were visualized by 1% agarose gel electrophoresis using SYBR Safe (Invitrogen, Darmstadt, Germany). Amplicons were eluted using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and sequenced for verification on a 3730xl DNA analyser (Macrogen Europe, Amsterdam, The Netherlands). Only *B. aphidicola* and *S. symbiotica* were detected in our aphid population and each individual harboured both bacterial symbionts (data not shown). The sequences were compared against NCBI databases using BLAST and deposited under accession numbers KX900450–KX900452 for *S. symbiotica* and KX910798–KX910801 for *B. aphidicola* [62].

4.3. Aphid Feeding with AMPs

A. pisum nymphs (48 h old) were fed for three days on an artificial AP3 diet in modified chambers [63,64]. The AP3 diet was mixed with the corresponding AMP or control treatment. Ten nymphs were placed in each chamber and five replicates were included per treatment. AMPs were tested at three different concentrations: 50, 250, and 500 µg/mL. Untreated aphids were fed on the control AP3 diet. Positive control treatments comprised aphids fed on the AP3 diet supplemented with the insecticide imidacloprid (5 µg/mL) or the antibiotic rifampicin (50 µg/mL) (Sigma-Aldrich, Taufkirchen, Germany) [30,64]. Imidacloprid is strongly hydrophobic, and was therefore prepared first as a highly concentrated stock (1000 µg/mL) in acetone and working solutions were diluted in the AP3 diet. The corresponding control (AP3 + acetone) was tested on the aphids, and survival was not affected when compared to AP3 diet alone or AP3 diet diluted with water (data not shown). Mortality was scored after 24, 48, and 72 h of feeding. Aphids that survived the three-day treatment were transferred to agar plates containing bean plant leaves and reared for another two weeks in order to determine the impact of the diets on survival and reproduction [65].

4.4. In Vitro Activity of Scorpion and Insect AMPs against Serratia Symbiotica CWBI-2.3

S. symbiotica strain CWBI-2.3, the only aphid symbiotic bacterium that can be cultivated in the laboratory, was purchased from the Leibniz Institute DSMZ (Braunschweig, Germany) and cultivated as recommended by the supplier. MICs were determined according to the CLSI guidelines using a broth microdilution assay in 96-well polypropylene microtiter plates. The bacteria were cultivated overnight at 28 °C using 535 medium (Tripticase soy broth) and diluted to 5×10^5 CFU/mL in broth. The AMPs were dissolved in water to a concentration of 4 mg/mL and a series of two-fold dilutions was prepared in 535 broths, ranging from 500 to 4 µg/mL. *S. symbiotica* CWBI-2.3 in an unmodified medium was used as a positive control, and blanks were prepared with medium only or with medium and water (the latter to exclude any possible negative effect of water on the bacteria). The bacteria were incubated for 18 h and the absorbance at 600 nm was recorded every 20 min. The MICs were defined as the lowest concentrations of AMP causing complete bacterial growth inhibition.

4.5. Relative Quantification of Bacterial Symbionts In Vivo

The density of the *B. aphidicola* and *S. symbiotica* populations in vivo was determined by qPCR. Genomic DNA was extracted from pools of five aphids, as previously described [60]. Three biological replicates were prepared per treatment. The primers used for the identification of bacterial symbionts and the reference genes are listed in Table S2 [66]. Amplifications were carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The reaction volume was 10 µL, comprising 2 µL of template DNA (25 ng/µL), 10 µM of each specific primer and 5 µL of SYBR Green PCR Master Mix (Applied Biosystems). Each reaction was heated to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis was performed by increasing the temperature from 60 °C to 95 °C for 15 s, cooling to 60 °C for 60 s and heating to 95 °C for 15 s. The expression of each gene was tested in triplicate to ensure reproducibility. Relative abundance values for each symbiont were calculated by comparing the threshold cycle (Ct) of each target gene to that of the aphid ribosomal protein L32 gene [67] and efficiencies were calculated using LinReg PCR software.

4.6. Localization of Bacterial Symbionts In Vivo by FISH

FISH was carried out as previously described [68], with slight modifications. Treated *A. pisum* nymphs were fixed for three days in Carnoy's solution (6:3:1 chloroform:ethanol:glacial acetic acid) and then bleached in 6% H_2O_2 in 96% ethanol for 1 week. After bleaching, samples were washed in 100% ethanol and then hybridized overnight in hybridization buffer (20 mM Tris-HCl pH 8.0, 0.9 M NaCl, 0.01% sodium dodecylsulfate, 30% formamide) containing 100 nM of each fluorescent probe and 500 nM DAPI. Different probes were used to label *Buchnera* (ApisP2a) and *Serratia* (SerratiaPA) as shown in Table S2 [15]. After hybridization, samples were rinsed three times with phosphate buffered saline containing 0.3% Triton X-100 and viewed under a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). We analysed a minimum of 20 samples from each treatment. The specificity of detection was confirmed using controls with no probe and specimens were pre-treated with RNase.

4.7. Data Analysis

All data were analysed using SPSS v17.0 software (SPSS Inc., Chicago, IL, USA) and statistical significance was defined as $p < 0.05$. For mortality assessment, we used non-parametric survival analysis (Kaplan-Meier) and multiple pairwise comparisons among different groups were carried out using log-rank tests to assess efficiency. The total number of offspring, time to reproduction, and relative numbers of bacterial symbionts were analysed using the Wilcoxon ranked sum test for non-parametric data and a paired *t*-test for parametric data.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/9/9/261/s1, Figure S1: Scheme representing the methodology followed; Table S1: Statistical data for Kaplan-Meier analysis shown in Figure 1; Table S2: List of primers and probes.

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Author Contributions: Karen Luna-Ramirez and Marisa Skaljac conceived and designed the experiments. Marisa Skaljac, Karen Luna-Ramirez, Jens Grotmann and Phillipp Kirfel performed the experiments. Karen Luna-Ramirez and Marisa Skaljac analysed the data. Andreas Vilcinskas contributed reagents, materials and analytical tools. Marisa Skaljac, Karen Luna-Ramirez and Andreas Vilcinskas wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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5.5 PATENT AND CONFERENCE PARTICIPATIONS

PATENTANMELDUNG

- Vilcinskas A., Skaljac M, **Kirfel P.** Methods of Multi-Species insect pest control Application number EP19209940

CONFERENCE PARTICIPATIONS

- **Insecta 2018**
  Gießen, Germany
  Oral presentation: *Inhibition of histone acetylation/deacetylation alters longevity, development and fecundity of the pea aphid*

- **XI European Congress of Entomology 2018**
  Naples, Italy
  Oral presentation: *Epigenetic inhibition impairs survival, development and fecundity of Acyrthosiphon pisum*

- **Insecta 2017**
  Berlin, Germany
  Poster: *Epigenetic inhibitors are affecting life traits of the pea aphid*

- **Ecology of Aphidophaga 13** (2016)
  Poster: *Effects of Scorpion Antimicrobial Peptides on Bacterial Symbionts of Aphids*

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VERSICHERUNG AN EIDES STATT

Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

_____ Bad Camberg, den 6. Februar 2021

(Phillipp Peter Kirfel)