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Strong impact of orally administered BCR4 defensin on aphid survival, embryo development, and symbiotic cells in three *Acyrthosiphon pisum* parthenogenetic lines

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Abstract

Aphids are major crop pests capable of colonizing the main plants grown for human consumption. They have specialized cells, the bacteriocytes, which house the obligate symbiont *Buchnera aphidicola* that provide them with essential nutrients missing from their diet. Bacteriocyte-specific cysteine-rich peptides (BCRs) are encoded by a defensin gene family exclusively present in aphids and specifically expressed in bacteriocytes. One BCR family member, BCR4, has been shown to have insecticidal properties against the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae). In the present study, we exposed the pea aphid to different doses of BCR4 and examined the impact on aphid survival, mass, anatomy, and fecundity, as well as on bacterial symbiosis. As different pea aphid lines with various symbiotic status may be differently affected by stress, we investigated the effect of BCR4 ingestion on three different *A. pisum* lines: LL01 and YR2-amp, that are mono-infected with *B. aphidicola*, and YR2-*Ri*, that is genetically identical to YR2-amp but also contains the extracellular facultative symbiont *Regiella insecticola*. Our results show a strong dose–response effect of BCR4 on LL01 survival and a more moderate effect on both YR2 lines, while an impact on the mass was observed in the three lines. Histological analyses revealed severe embryonic developmental defects due to the treatment. Finally, BCR4 treatment reduced symbiont quantity, with *B. aphidicola* being more affected than *R. insecticola*. This study supports the hypothesis that BCR4 could act as a key regulator of aphid symbiosis and development and highlights its potential as a candidate bioinsecticide for pest control.

Keywords Aphididae · Bacteriocyte-specific cysteine-rich peptide · Bio-insecticidal peptide · Symbiont · Pest management

Key message

- BCR4 belongs to a new defensin family of peptides whose role in aphid symbiosis remains elusive
- BCR4 ingestion affects aphid survival, weight, and embryo development in a dose-dependent manner
- Susceptibility of aphids to BCR4 varies with the genetic background and symbiotic status
- BCR4 lowers symbiont populations, affecting Buchnera aphidicola more than Regiella insecticola
- BCR4 is a promising insecticide candidate for aphid management

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Introduction

Aphids are major crop pests, causing global losses estimated at hundreds of millions of dollars each year (Gula 2023). They make up to 26% of pests on important food crops in temperate regions, including maize, wheat, potatoes, sugar beets, barleys, and tomatoes (Calevro et al. 2019; Dedryver et al. 2010). These hemipteran insects feed on plant phloem sap, causing physiological stress that hampers plant growth and leads to yield losses (Rabbinge et al. 1981; Dedryver et al. 2010; El Fakhouri et al. 2021; Luo et al. 2022). Aphids have also been reported as the most efficient and important vectors of plant viruses, transmitting nearly 300 different virus species (Hogenhout et al. 2008), which corresponds to 30% of the total number of plant pathogenic viruses identified to date.

Aphids'ecological success is largely due to their symbiotic relationship with the γ-proteobacterium *Buchnera aphidicola*, an obligate 'primary' endosymbiont that provides them with essential nutrients poorly represented in their diet (Wernegreen 2012; Hansen & Moran 2014). This primary symbiont is vertically transmitted and lives within specialized host cells known as bacteriocytes (Baumann 2005) that are the interface of metabolic exchanges between the symbiotic partners (Smith & Moran 2020), regulate symbiont populations (Simonet et al. 2018) and are involved in their vertical transmission (Koga et al. 2012). Aphids can also host various facultative 'secondary' endosymbionts, including *Spiroplasma*, *Wolbachia*, *and Rickettsia* species, as well as Enterobacteria such as *Regiella insecticola*, *Hamiltonella defensa*, and *Serratia symbiotica* (Oliver et al. 2010; Guo et al. 2017). These secondary symbionts, located in the hemolymph, secondary bacteriocytes, and/or other organs (sheath cells, oenocytes, and digestive tract) (Tsuchida et al. 2014), can be transmitted both vertically and horizontally (Darby & Douglas 2003; Li et al. 2018; Pons et al. 2019a, b; Renoz et al. 2019). They have been associated with benefits like thermal resistance (Montllor et al. 2002), protection against natural enemies (Oliver et al. 2003; Scarborough et al. 2005; Frago et al. 2017), and adaptation to different host plants (Renoz 2024; Tsuchida et al. 2004).

Current aphid control strategies rely essentially on chemical treatments, whose extensive use contributes to environmental pollution and the development of insect resistances (Goulson 2018). Consequently, there is an urgent need for the development of environmentally-friendly alternatives. Small disulfide-rich proteins (DRPs) from plants or arthropods are promising candidates to be used instead of, or in combination with low doses of, chemical insecticides, due to their wide range of biological activities related to host defense, as well as their stability and resistance to enzymatic degradation (Ho et al. 2023).

A specific class of DRPs, known as bacteriocyte-specific cysteine-rich (BCR) peptides, has been identified in the pea aphid, *Acyrthosiphon pisum* (Shigenobu & Stern 2013). These peptides, encoded by seven orphan genes, are expressed in the bacteriocytes of both embryonic and adult aphids, suggesting a role in the maintenance of bacteriocyte homeostasis and the control of endosymbionts (Shigenobu & Stern 2013). Previous research has shown that BCR4, in particular, exhibits insecticidal effects when orally delivered to the *A. pisum* line LL01, reducing its survival and mass (Loth et al. 2022).

This investigation aimed to determine what the developmental and anatomical bases of BCR4 toxicity are, whether this toxicity could be explained by a direct effect on symbiotic cells, and whether those vary depending on the aphid genetic background and/or the presence of facultative symbionts. The effects of ingesting sublethal doses of BCR4 were investigated using a panel of complementary approaches (life history traits, histological and FISH analyses, and flow cytometry) applied to three *A. pisum* lines: LL01, YR2-amp, and YR2-*Ri*. LL01 and YR2 lines are genetically different. Moreover, the first two lines only harbor the obligate symbiont *B. aphidicola*, while the third also hosts the facultative symbiont *R. insecticola*.

Material and methods

Aphid lines and rearing

All the experiments were performed using the pea aphid, *Acyrthosiphon pisum*. Three distinct lines were used: LL01, YR2-*Ri*, and YR2-Amp. LL01 corresponds to a natural line (Rahbé & Febvay 1993) harboring only the obligate primary symbiont *B. aphidicola*. YR2-*Ri* naturally harbors both *B. aphidicola* and the facultative symbiont *R. insecticola* (Simon et al. 2011). YR2-Amp was produced from the YR2-*Ri* line, after treatment with antibiotics to eliminate *R. insecticola* (Koga et al. 2007; Simon et al. 2011). The presence of the latter and the absence of other secondary symbionts that may be found in pea aphids (*i.e., Hamiltonella defensa*, *cd. Fukatsuia symbiotica*, *Rickettsia* sp, *Rickettsiella* sp, *Serratia symbiotica* and *Spiroplasma* sp.) were verified by a PCR-based diagnostic developed by Peccoud et al. (2014). The three lines were maintained as strictly parthenogenetic matrilines on young broad bean plants (*Vicia faba*, L. cv. Aquadulce), with a photoperiod of 16 h light-8 h dark, at 21 °C with ~60% of humidity. To obtain synchronized aphids, around 100 reproductive females (9–11-day- old) per line were left on plants for 24–72 h (pre-synchronization). The adults were then removed, and newborn nymphs were kept on the plant for 9 days (= adult stage), then transferred to a new plant for

24 h (synchronization). The resulting synchronized first instar nymph (< 1 day old) were then placed on artificial diets containing different concentrations of the BCR4 peptide for the different experiments. The complete experimental design is illustrated in Fig. S1.

Synthesis and purification of BCR4

The BCR4 peptide sequence was synthesized and folded according to the optimized procedure described in a previous study (Loth et al. 2022). Briefly, BCR4 was generated by native chemical ligation (NCL) of two peptide segments, followed by oxidative folding to form the three disulfide bridges. Both peptide segments were purified using C18 reverse phase (RP) HPLC. The purified peptides were then chemoselectively coupled and purified under standard NCL conditions to yield the pure reduced form of BCR4. This form was then subjected to in vitro oxidative folding followed by purification to homogeneity using C18 RP-HPLC. Analysis of the final folded product by ESI-HRMS confirmed the presence of three disulfide bridges.

Survival and growth monitoring following BCR4 ingestion

To test the effect of BCR4 ingestion on aphid survival and growth, the synthetic peptide was serially diluted in AP3 artificial diet (Rahbé et al. 1988; Calatayud 2000) at the following concentrations (in µg/ml): 0 (control), 50, 100, 250, 375, 500, 750 and 1000, and prepared in ad hoc feeding chamber as previously described (Rahbé et al. 1988). For each aphid line and each concentration, three groups of ten first instar nymphs were collected and fed on independent feeding chambers. In order to avoid contamination, the artificial diet was replaced on the fourth day of treatment. Moreover, to avoid crowding of the feeding chamber, aphids were split into two groups of five aphids (Fig. S1). Survival was monitored daily for seven days. At the end of the experiment, all surviving aphids in the control groups had reached the fourth nymphal stage and ten surviving aphids per group were randomly selected and individually weighed on a Mettler AE163 analytical microbalance (Mettler Toledo, Columbus, OH, United States). To confirm the specificity of the observed effect, i.e. that this was due to BCR4, the same experimental design was repeated, using a negative control peptide at the highest concentration only (1000 µg/ml). The peptide used (U_{11}; for details, see Barassé et al. 2023) comes from the ant *Tetramorium bicarinatum*, possesses a disulfide bond and a molecular weight of 4.0 kDa, close to the 5.9 kDa of BCR4.

Analysis of the effect of BCR4 ingestion on aphid morphology and anatomy

To study the effect of BCR4 ingestion on aphid morphology and anatomy, the previous experimental design was repeated with aphids fed either on a control AP3 diet or on a diet supplemented with the dose of 1000 µg/ml of BCR4 for 7 days. At this time, all aphids in the control groups had reached the fourth nymphal stage. Dissection and histological preparations were carried out as described below.

Dissection and macroscopic observations

After 7 days of treatment, at least six aphids per condition and per line were randomly selected, and photographed with a Moticam S6 camera (MoticEurope, Barcelona, Spain) attached to a Leica MZ FLIII stereomicroscope (Leica Microsystems, Wetzkar, Germany) to compare their size, shape and color. They were then dissected in isoosmotic buffer A (0.025 M KCl, 0.01 M MgCl2, 0.25 M Sucrose, and 0.035 M Tris–HCl, pH 7.5) by opening the abdomen with microdissection tweezers (Dumont type 5 Dumoxel; Electron Microscopy Science, Hatfield, UK) as previously described (Ribeiro Lopes et al. 2021). The digestive tract and the embryonic chains were isolated and photographed using the same camera.

Aphid section preparation

For histological staining and FISH (Fluorescent In-Situ Hybridization) of treated and control aphids, four individuals per condition were randomly selected after 7 days of treatment, and sections were prepared as previously described (Ribeiro Lopes et al. 2022). Briefly, antennae and legs were removed from aphids with microdissection tweezers, before immersing them in a solution of PBS containing 4% paraformaldehyde and 0.1% Triton X-100 at 4 °C for 24 h. Aphids were then transferred to a solution of PBS with 4% paraformaldehyde. After several washing steps in PBS, aphids were embedded in 1.3% agar. The resulting samples were dehydrated through a series of ethanol solutions ranging from 70 to 100%, and transferred in 1-butanol at 4 °C for at least 24 h. In order to prepare aphid sections, aphids were embedded in Paraplast/1-butanol (v/v) for 10 h and then in 100% Paraplast (Paraffine Paraplast Plus Mc Cormick Scientific Dutscher). Resulting Paraplast blocks were sectioned at 3 µm thickness using a HM340E rotary microtome (ThermoScientific, Waltham, MA, United States). Sections were positioned on polylysine coated slides and conserved at 4 °C until staining.

Histological preparation and observation

Sections were prepared for Hematoxylin and Eosin (H&E) staining with RAL products (RAL Diagnostics, Martillac, France) using a protocol adapted from Sapountzis et al. (2014). Briefly, sections were deparaffinated in methylcyclohexane (2 × 10 min) and rehydrated through an ethanol series to PBS. Nucleus and cytoplasm were stained in Mayer Hematoxylin for 3 min and Eosin for 2 min, respectively, with a water rinse in between. Sections were then washed in water and treated through an ethanol series from 70 to 100%. After a rapid bath in Diasolv (Diapath, Martinengo, Italia) solution, sections were mounted with Diamount mounting medium (Diapath, Martinengo, Italia). Histological observation was performed under transmitted light, on a Thunder Imager 3D Live Cell™ microscope (Leica, Wetzlar, Germany) with 4X or 20X magnification.

FISH analysis

FISH procedure was adapted from Simonet et al. (2018). Sections were deparaffinated using methylcyclohexane (2 × 10 min), followed by two 10 min baths in 100% ethanol, and then air-dried. Deproteinization was conducted with 0.01 M hydrochloric acid and 10 mg/mL pepsin at 37 °C for 10 min. Sections were rinsed in PBS and dehydrated through a series of ethanol solutions ranging from 70 to 100%. Prehybridization took place at 45 °C for 30 min in a prehybridization buffer consisting of 79% hybridization buffer (0.9 M NaCl, 20 mM Tris, 5 mM EDTA, pH 7.2), 20% Denhardt's solution (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA in 500 mL water), and 1% SDS solution (10% SDS). In situ hybridization was performed by incubating the slides at 45 °C for 3 h, shielded from light, with 5 µl of 100 µM oligonucleotide probes specifically targeting *B. aphidicola* and *R. insecticola* 16S rRNAs (Table S1). Probes were prepared in prehybridization buffer and their 5'end was labeled with Alexa Fluor 488 and Alexa Fluor 594, respectively. Sections were washed twice in washing solution (99% hybridization buffer and 1% SDS 10%), rinsed in PBS and finally mounted using PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific) containing DAPI (3 µg/mL; Vector Laboratories). Positive fluorescent signals were analyzed using a Thunder Imager 3D Live Cell™ microscope (Leica) equipped with the appropriate emission filters.

Analysis of the effect of BCR4 ingestion on aphid symbiont

The BCR4 bioassay experiment was repeated with only aphids fed either a control AP3 diet or a diet supplemented with of 1000 µg/µl BCR4 for 7 days. To assess the impact of the treatment on the quantity of the primary symbiont *B. aphidicola* and the secondary symbiont *R. insecticola,* a flow cytometry approach, that proved to be effective to obtain the absolute numbers of bacteria in this model, was used (Simonet et al. 2016).

For each condition, symbiotic bacteria from 12 whole aphids were purified as previously described (Simonet et al. 2016). Aphids were gently crushed with a Potter homogenizer in 3 ml of ice-cold buffer A and the homogenate was consecutively filtered through nylon net filters with 60, 30 and 10 µm pore sizes (Merck Millipore, Tullagreen, Ireland). Following centrifugation (4,000 × g, 5 min, 4 °C), the pellet of endosymbiotic bacteria was re-suspended in 200 µl, for aphids reared on AP3 + BCR4 (1000 µg/ml) medium, or 400 µl, for aphids reared on AP3, of NaCl 0.85% supplemented with 20% glycerol as a cryoprotective agent, and stored at − 80 °C until flow cytometry analysis. Immediately prior to flow cytometry analyses, samples were thawed for 3 min at 42 °C, vortexed and diluted to 1:20 with a NaCl 0.85% solution to reach the optimal cell concentration for flow cytometry analysis (< 2,000 events/sec). All solutions were filter-sterilized at 0.22 µm prior to use to avoid contamination with exogenous bacteria. For bacterial staining, 0.5 µl of the nucleic acid probe SYTO9 (3.34 mm; Molecular Probes Inc, Eugene, OR, USA) was added to 300 µl of the diluted bacterial suspensions. The mixture was then vortexed and incubated in the dark for 15 min at room temperature, following the manufacturer's instructions. The stained samples were analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a blue laser (488 nm, air-cooled, 20 mW solid state) and cell green fluorescence was acquired with a photomultiplier tube detector and a 530 nm band-pass filter (503–563 nm). Flow cytometry measurements were run at low flow rate (14 µl/min) and the core stream was allowed to stabilize for 30 s prior to the 2 min acquisition. Data were collected and processed using BD Accuri C6 software (version 1.0.264.21, BD Biosciences). The analyses were performed using logarithmic gains and specific detector settings, adjusted on non-stained samples to eliminate endosymbiont autofluorescence. Then, the endosymbiont population was identified by screening samples on FSC-W versus FSC-H (Forward Scatter Width vs. Height) and SSC-W versus SSC-H (Side Scatter Width vs. Height) dot plots. *B. aphidicola* populations were identified using the same parameter than previously described (Simonet et al. 2016). A second population of SYTO9-positive event, of different size and granularity, was identified in YR2-*Ri* samples and used to count *R. insecticola* symbionts (Fig. S2). For each condition, four biological replicates were processed.

Statistical analysis

Statistical analysis and graphical representations were conducted using the R software (v4.3.1) (R Core Team 2023). R packages « survival» and « survminer» (Therneau 2024) were used to analyze survival data, comparing curves with a Wilcoxon-Gehan rank test. To estimate the lethal time taken for 20% of the aphid population to die (LT_{20}) when fed on artificial diet containing 1000 µg/µl of BCR4, generalized linear models (glm) with a probit link transformation were fitted for each aphid line and compared with a ratio test, using the R package "ecotox" (Hlina et al. 2021). Dose–response curves of the effect of BCR4 ingestion on aphid mass were fitted with the « drc» package (Ritz et al. 2015) using a log-logistic model with three parameters (with the lowest limit fixed to 0) and box-cox transformation, allowing to predict 95% confidence intervals (CI_{95}%) and the effective dose that affect 50% of the mass compared to control (ED_{50}).

For the flow cytometry analyses, the absolute numbers of symbionts obtained for each aphid pool were converted into "symbiont numbers per aphid". A linear regression model was applied on data. Pairwise comparisons were performed to investigate significant differences between modalities using the eammeans function of the R package "emmeans".

Results

BCR4 treatment reduces survival and mass of three aphid lines

The dose–response effect of BCR4 ingestion on survival was first compared between the three aphid lines LL01, YR2-amp, and YR2-*Ri*. In the absence of BCR4, the three lines show comparable survival rates on the artificial diet, with a 7-day survival of 93%, 97%, and 97% for LL01, YR2-amp, and YR2-*Ri*, respectively. Survival of LL01 and YR2-amp lines is significantly affected by BCR4 ingestion (log-rank test: $p < 0.001$), but not that of YR2-*Ri* (log-rank test: $p = 0.113$) (Fig. 1). However, both YR2 lines survive better than the LL01 line (log-rank test LL01 vs YR2-amp or YR2-*Ri*: $p < 0.001$). Similar results were obtained when focusing solely on the treatment with the highest concentration of BCR4 (1000 µg/µl): (i) the 7-day survival is of 77% and 83% for YR2-amp and YR2-*Ri*, respectively, and only 50% for LL01; (ii) the estimated time to reach 20% of the mortality

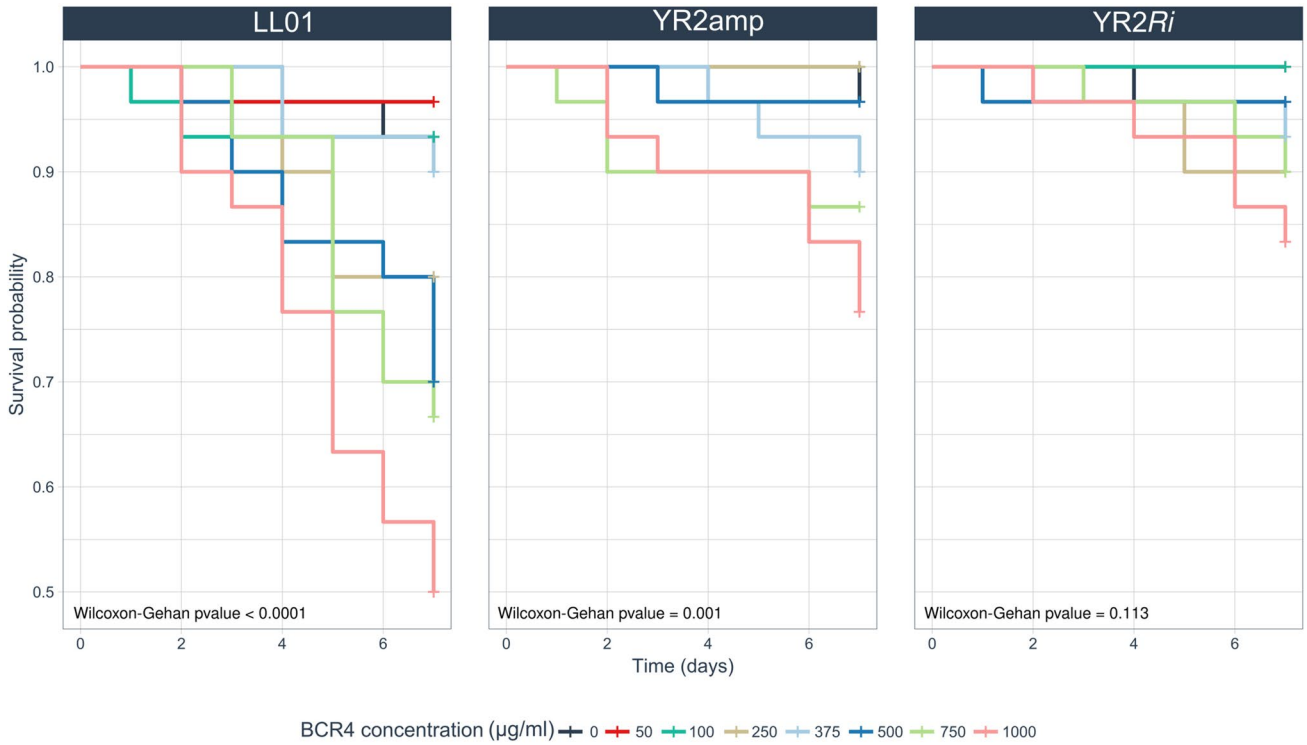

Fig. 1 Dose–response effect of BCR4 ingestion on survival of three *Acyrthosiphon pisum* lines. Survival curves of aphid fed on artificial diets (AP3) containing different concentrations of BCR4 peptide during 7 days. Left, central, and right panels correspond to survival curves of LL01, YR2-amp, and YR2-*Ri* lines, respectively. $n = 30$ for each BCR4- oncentration group for each line. Data were analyzed using the Wilcoxon–Gehan Test

(LT_{20}) for LL01 (3.5 days) is significantly different from the LT_{20} of both YR2-amp (6.8 days; ratio test: $p = 0.002$) and YR2-*Ri* (8,6 days; ratio test: $p < 0.001$). In contrast, no mortality was observed in aphids fed with 1000 µg/ml of U11 peptide (Fig. S3).

The results show that there is a dose–response effect of BCR4 on the mass of each line (lack-of-fit test: $p < 0.05$; Fig. 2a). Feeding on BCR4 has a significant, negative impact on aphids' mass, with an average reduction of 78%, 65%, and 75% for LL01, YR2-amp, and YR2-*Ri*, respectively, compared to the control. The BCR4 concentration that would cause a 50% reduction in aphid mass (ED_{50}) was predicted, revealing a more potent effect on the LL01 line ($ED_{50} \pm$ SD of 383.8 ± 39.6 µg/ml; 535.3 ± 75.6 µg/ml; and 445.2 µg/ml ± 52.1 for LL01, YR2-amp, and YR2-*Ri*, respectively). Discoloration of the aphid cuticle is also observed for each line, with a more noticeable effect on YR2-amp and -*Ri* compared to LL01 probably due to their more contrasted color (Fig. 2b–d).

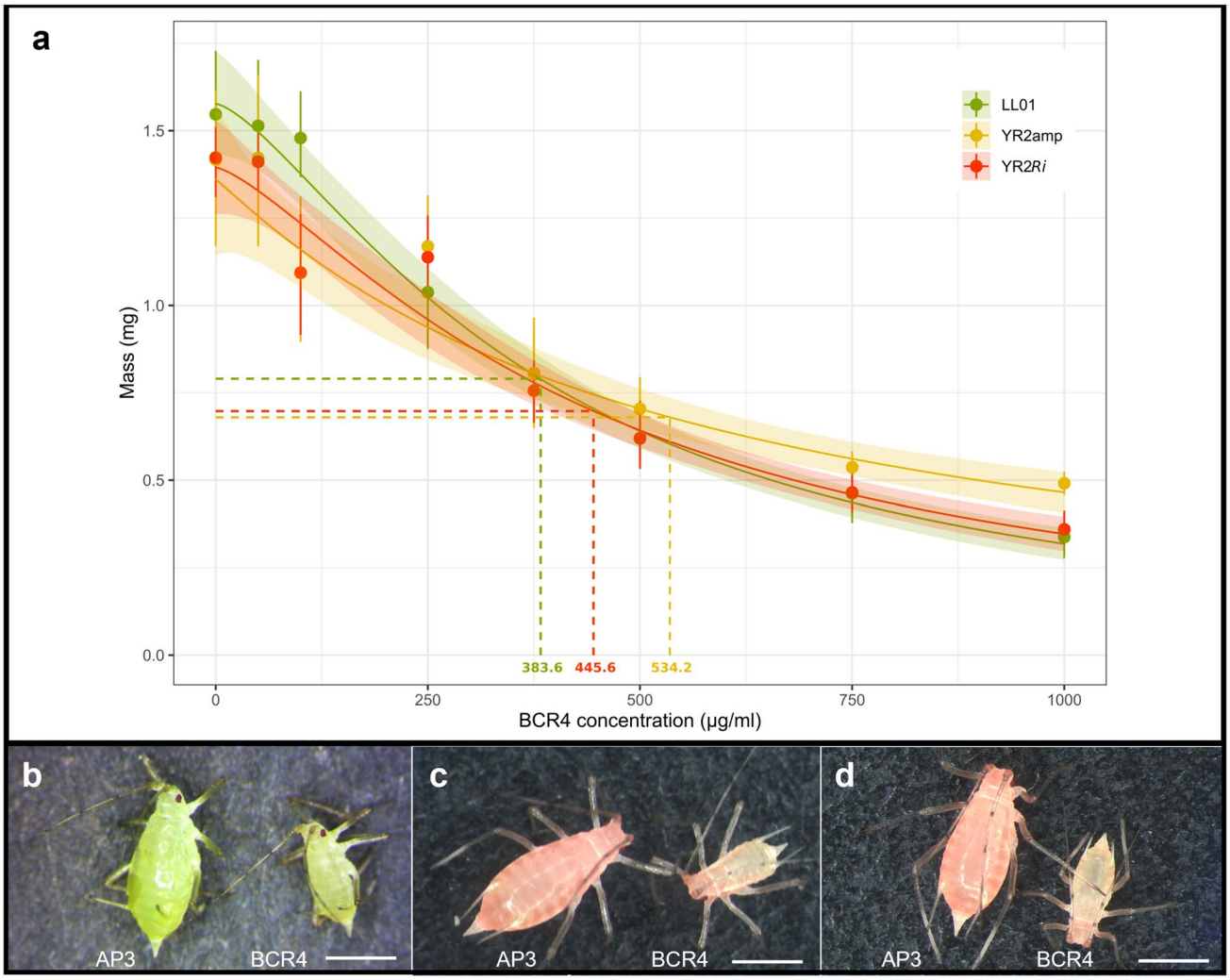

Fig. 2 Effect of BCR4 ingestion on fresh mass, size, and body color of three *Acyrthosiphon pisum* lines. **a** Dose-reponse curves of aphids' mass following 7 days of feeding on artificial diets containing different concentrations of BCR4 peptide. Results for the LL01 line, YR2-amp line, and YR2-*Ri* line, are presented in green, yellow, and red, respectively. For each BCR4 concentration, dots with error bar correspond to mean mass with standard deviation. For each line, dose response effect of BCR4 ingestion was modeled with a log-logistic model with three parameters followed by box-cox transformation and displayed with its predicted confidence interval. Dash lines correspond to the estimated BCR4 concentration that leads to a 50% reduction in mass compared with aphids fed on AP3 medium without BCR4 (ED50). $n = 10$ aphids for each BCR4 concentration group for each line. **b–d** Pictures of seven-day-old aphids fed on artificial diets (AP3) complemented or not with 1000 µg/ml of BCR4 during 7 days. Panels b, c, and d correspond to the LL01 line, YR2-amp line, and YR2-*Ri* line, respectively. Scale bar: 1 mm

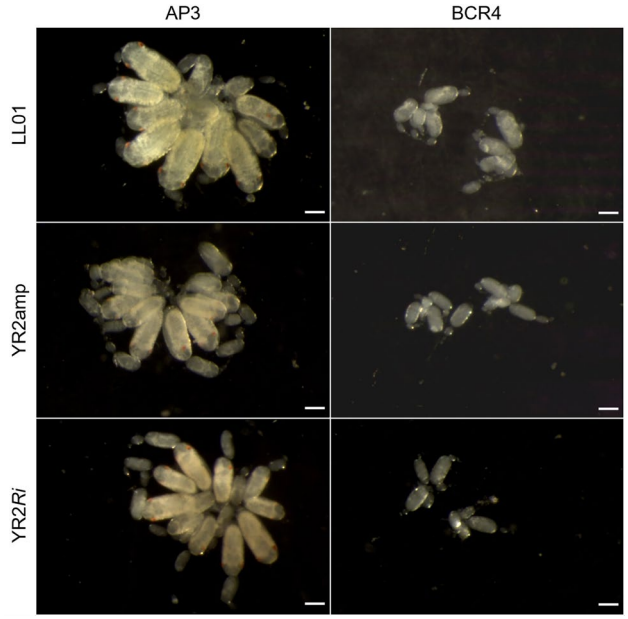

Fig. 3 Effect of BCR4 ingestion on dissected embryonic chain in the three *Acyrthosiphon pisum* lines**.** Left panels correspond to embryonic chains dissected from LL01, YR2-amp and YR2-*Ri* lines fed on AP3 medium (control) for 7 days. Right panels correspond to embryonic chains dissected from LL01, YR2-amp, and YR2-*Ri* fed on BCR4 diluted in AP3 medium (treated) for 7 days. Scalebar: 200 µm

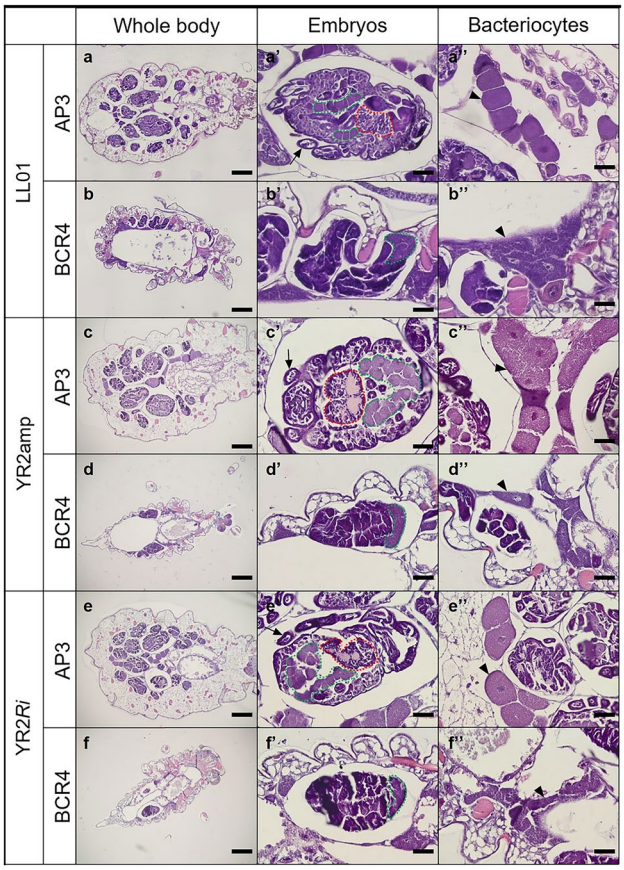

Fig. 4 Effect of BCR4 ingestion on embryos and bacteriocytes in three *Acyrthosiphon pisum* lines. **a–f** H&E staining of 7-day-old aphids belonging to the LL01, YR2-amp, and YR2-*Ri Acyrthosiphon pisum* lines fed on artificial diets (AP3) complemented or not with 1000 µg/ml of BCR4 during 7 days. **a'–f'** Higher magnification of embryos. The green- and red-dotted lines surround the bacteriocytes and the central nervous system of embryos, respectively. The black arrows indicate a visible limb (antenna or leg). **a''–f''** Higher magnification of maternal bacteriocytes indicated by the black arrowheads. Scalebars: 100 µm in *a-f*, 20 µm in *a'-f'*, 20 µm in *a''-f''*

Impact of BCR4 on the anatomy of aphids and their embryos

To assess whether the reduction in mass following BCR4 treatment was accompanied by changes in aphid tissue organization, dissections, histological and FISH analyses of individuals treated with the highest concentration of BCR4 were carried out.

Macroscopic observations

Macroscopic observations of dissected aphids revealed no obvious differences between the digestive tracts of control and treated individuals. No signs of fragility were observed during dissection, and the different regions of the digestive tract (foregut, midgut, hindgut) were clearly identifiable and appeared intact in both aphid groups (Fig. S4). However, significant differences were observed in the development of embryonic chains (Fig. 3). In control aphids, the classic two ovaries, each containing seven ovarioles, are clearly visible. These ovarioles consist of chains of embryos at different developmental stages. In these aphids, the most developed embryos exceed 500 µm in length and are pigmented. The pair of red eyes and the beginning of thorax segmentation are also visible (Fig. 3). In contrast, the embryonic chains of treated individuals are more crumbly and fragile. They contain fewer and smaller embryos, with the largest ones reaching only 300 µm. In addition, no coloration, no eyes, and no segmentation is noticeable, regardless of the aphid line (Fig. 3).

Histological analysis with hemalum and eosin staining

Histological analyses confirmed the macroscopic observations. Specifically, H&E staining reveals no evidence of epithelial cell degradation in the digestive tract following the treatment (Fig. 4d or f). On the other hand, an adverse effect of BCR4 on aphid development is clearly visible. In particular, numerous well-developed embryos with a developed central nervous system (CNS) and visible limbs (Fig. 4 a', c', e') can be distinguished in sections from control aphids. Bacteriocytes are cellularized and form two

lobes located in proximity to the developing digestive tract. While embryos are still visible in treated aphids, they are negatively impacted by the treatment: their organs are indistinguishable and the bacteriocytes, though present, appear acellularized, with no apparent cell membrane (Fig. 4 b', d', e'). In all the embryos that could be observed, the bacteriocyte mass is found at the posterior pole, which suggests that these embryos are undergoing a developmental delay.

BCR4 ingestion also shows a strong effect on the morphology of the mother's bacteriocytes (Fig. 4 a"–f"). Although the symbiotic cells are present in both control and treated aphids, bacteriocytes from the latter are deformed, appearing crushed between organs, and sometimes difficult to distinguish from each other (Fig. 4b", d", e"). In addition, observation of whole-insect sections suggests a reduction in bacteriocyte number (Fig. 4a-f).

FISH analyses

FISH analyses were carried out to assess the effect of BCR4 on symbiont localization and the structure of bacteriocytes. Observation of control aphids (reared on AP3 artificial diet) shows that the localization of *B. aphidicola* and *R. insecticola* is the same as previously described in other aphid lines (Moran et al. 2005; Tsuchida et al. 2005): *B. aphidicola* is exclusively found in maternal and embryonic bacteriocytes (Fig. 5a-c), while *R. insecticola* is observed extracellularly, in the hemolymph and around the bacteriocytes, and intracellularly, in sheath cells attached to the bacteriocytes, resulting in a signal that completely surrounds the bacteriocyte clusters (Fig. 5c).

After BCR4 treatment, the localization of the primary symbiont is not affected, and it is strictly confined to maternal and embryonic bacteriocytes. Nevertheless, consistent with H&E observations, maternal bacteriocytes appear deformed and less numerous (Fig. 5a'–c'). Moreover, in embryos, bacteriocytes appear to be packed in the posterior region, in contrast to their classical localization around the digestive tract (Fig. 5d–f). Regarding *R. insecticola*, the signal is also detected in treated aphids at the same localization as in the control (Fig. 5f). However, a reduced signal is observed in the hemolymph and around the bacteriocytes and the signal surrounding the bacteriocyte clusters is not continuous as in the controls (Fig. 5c and f).

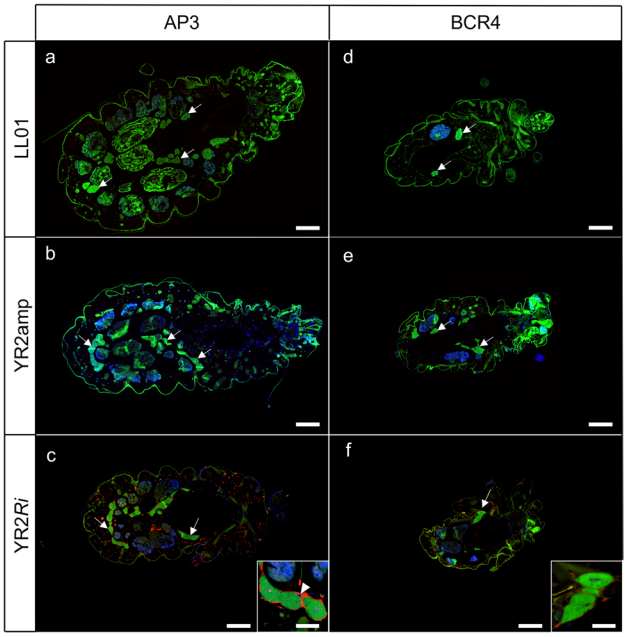

Fig. 5 Effect of BCR4 treatment on symbiont localization. FISH observation of 7-day-old aphids belonging to the LL01, YR2-amp, and YR2-*Ri Acyrthosiphon pisum* lines fed on artificial diets (AP3) only (**a–c**) or complemented with 1000 µg/ml of BCR4 during seven days (**d–f**). *Buchnera aphidicola* is labeled in green and *Regiella insecticola* in red, respectively. Maternal bacteriocytes are indicated with white arrows. In YR2-*Ri* individuals (**c**, **f**) higher magnification shows maternal bacteriocytes filled with *B. aphidicola* and surrounded by *R. insecticola*. White arrowhead indicates *R. insecticola* signal in a sheath cell. Scale: 200 µm and 50 µm for whole body images and zoom on bacteriocytes, respectively

BCR4 significantly reduces symbiont populations

To assess whether the observed effect of BCR4 ingestion on bacteriocyte morphology and numbers translates into a change in the density of symbiont populations, a flow cytometry analysis using a single-cell counter cytometer was performed to determine the absolute number of symbionts per aphid (Simonet et al. 2016). The mean total number of events corresponding to *B. aphidicola* decreases drastically from 1.07×10^6 to 1.48×10^5 for LL01, from 6.67×10^5 to 1.82×10^5 for YR2-amp, and from 7.04×10^5 to 1.10×10^5 for YR2-*Ri*. This corresponds to reductions of 86%, 73%, and 84%, respectively. The BCR4 treatment ($F_{1,17} = 120.172$, $p < 0.001$) and the interaction between line and treatment ($F_{2,17} = 4.224$, $p = 0.03$) significantly impact *B. aphidicola* count while the line alone do not ($F_{2,17} = 3.327$, $p = 0.06$). The mean total number of events corresponding to *R. insecticola* in YR2-*Ri* line decreases more modestly, from 1.79×10^6 to 7.57×10^5, a significant reduction of 58% ($F_{1,5} = 50.336$, $p < 0.001$) (Fig. 6).

Discussion

This study investigated the impact of BCR4 peptide ingestion on three *A. pisum* lines, LL01, YR2-amp, and YR2-*Ri*, differing in their genetic background (LL01 or YR2) and by the absence (LL01 and YR2-amp) or presence (YR2-*Ri*) of the secondary symbiont *R. insecticola*. Overall, we showed an insecticidal effect of BCR4, with a strong impact

on survival, fresh weight, embryo development, bacteriocyte morphology, and symbiont numbers.

Our findings corroborate previous results obtained by Loth et al. (2022), which showed a dose–response effect of BCR4 on aphid survival and weight. However, the latter study was carried out on a single genetic line (LL01), carrying only the primary symbiont *B. aphidicola*, whereas several works have shown a significant impact of the genotype on aphid response to biotic stresses, such as host plant changes (Ferrari et al. 2007), parasitoid wasp attacks (Libbrecht et al. 2007; Martinez et al. 2018), or temperature increase (Jahan et al. 2023), highlighting the importance of considering different genetic lines. In the present study, we showed a contrasting effect of BCR4 on aphids' survival depending on their genetic background: at equivalent concentration, LL01 individuals died faster and to a greater extent than YR2-amp and YR2-Ri. Similarly, BCR4 ingestion reduced aphid mass in a dose-dependent manner, with the LL01 line being the most affected. While the insecticidal mode of action of BCR4 is unknown to date, our findings on the impact of BCR4 on aphid survival are similar to previous results following the use of other known antimicrobial peptides on aphids. Indeed, ingestion of indolicidin (an AMP produced by bovine neutrophils) reduces the survival of *Myzus persicae* (Le-Feuvre et al. 2007) and Luna-Ramirez et al. (2017) showed that ingestion of certain scorpion AMPs exerts insecticidal activity against *A. pisum*.

Presence of *R. insecticola* had no impact on *A. pisum* survival, however, the symbiont aggravated the negative impact of BCR4 on aphid weight. Secondary symbionts have been reported to positively or negatively affect aphids' fitness, depending on the environment or the stress to which the insect is subjected, including xenobiotic exposure (Lemoine et al. 2020; Zytynska et al. 2021). For instance, infection by *Serriatia symbiotica* in *A. pisum* (Skaljac et al. 2018) and *Rickettsia* in the whitefly *Bemisia tabaci* (Kontsedalov et al. 2008), is associated with increased susceptibility to chemical insecticides, while the infection by *Hamiltonella defensa* is associated with reduced susceptibility in the aphid *Sitobion miscanthi* (Li et al. 2021). In *A. pisum*, infection by *R. insecticola* is associated with reduced aphid survival during heat stress (Russel and Moran, 2005) or bacterial infection (Luo et al. 2021). Conversely, the symbiont confers protection against fungal (Scarborough et al. 2005; Parker et al. 2013, 2017) or viral infections (Higashi et al. 2023), indicating that the bacterium could contribute to host defenses in different ways. Although symbionts have been reported to increase their hosts' sensitivity to insecticides (Kontsedalov et al. 2008; Skaljac et al. 2018), the underlying mechanisms remain unknown. Hosting secondary symbionts is a finely-tuned cost–benefit trade-off for aphids, as symbionts are associated with a permanent physiological cost, acting as sinks for metabolites (Zytynska et al. 2021). Harboring the *R. insecticola* symbiont has been shown to negatively impact the host fitness (Sochard et al. 2021; Man et al. 2023). Here, the cost of hosting *R. insecticola* may be exacerbated when *A. pisum* experiences stressful conditions such as exposure to BCR4. Similarly, specific symbiont combinations may aggravate the whitefly *B. tabaci* performance on unfavorable host plants (Benhamou et al. 2021).

The negative impact of BCR4 on survival and weight of *A. pisum* could be direct, by affecting aphid cells, or indirect, by targeting symbionts and disrupting the obligate nutritional relationship between the insect and its symbiotic bacteria. Concerning the direct cytotoxic effect of BCR4 on aphid cells, it is important to underline that this could not be excluded, as it has been shown that high concentration of certain AMPs could exert a cytotoxic activity, in vitro, against mammalian cells, by disrupting cell membranes (Vaucher et al. 2010; Greco et al. 2020). Moreover, eukaryotic cells are generally resistant to AMPs. Contrary to prokaryotes, eukaryotic cell membranes contain sterol and mainly zwitteronic phospholipids that stabilize the membrane and confer a protection against AMPs (Mason et al. 2007; Anderson et al. 2016; Almeida et al. 2021). Nevertheless, Greco et al. 2020 have shown that, when inoculated in rats, AMPs lose their cytotoxic potential.

To gain an in-depth understanding of the effects of BCR4 ingestion on aphids and their symbionts, dissection and histological observations were performed. These analyses revealed a similar and significant negative effect on the embryos of each aphid line. Following treatment, embryonic chains were more fragile and contained smaller and fewer embryos, for which it was impossible to distinguish appendages or organs on histological sections. Histological and FISH observations revealed that the bacteriocytes and the *B. aphidicola* they contain were mostly grouped at the posterior pole of the embryo, a localization that is usually limited to the early stages of embryo development (Miura et al. 2003; Braendle et al. 2003). Furthermore, embryonic bacteriocytes in treated aphids appeared acellularized, a disposition that is usually observed at the beginning of embryonic development. Taken together, these observations demonstrate a delay in bacteriocytes morphogenesis. Whereas previous studies using indolicidin (Le-Feuvre et al. 2007) or scorpion AMPs (Luna-Ramirez et al. 2017) have shown an adverse impact of these peptides on aphid fecundity, our study presents for the first time a disruption of aphid embryonic development caused by the ingestion of an AMP.

Moreover, analyses of FISH and H&E staining revealed an alteration in maternal bacteriocytes. While bacteriocytes in the control group are numerous and round-shaped, most bacteriocytes from treated aphids appear fewer in number and distorted in the three aphid lines. The effect on aphid bacteriocytes is similar to the one observed by Le-Feuvre et al. (2007) in *M. persicae*, who reported distortion of

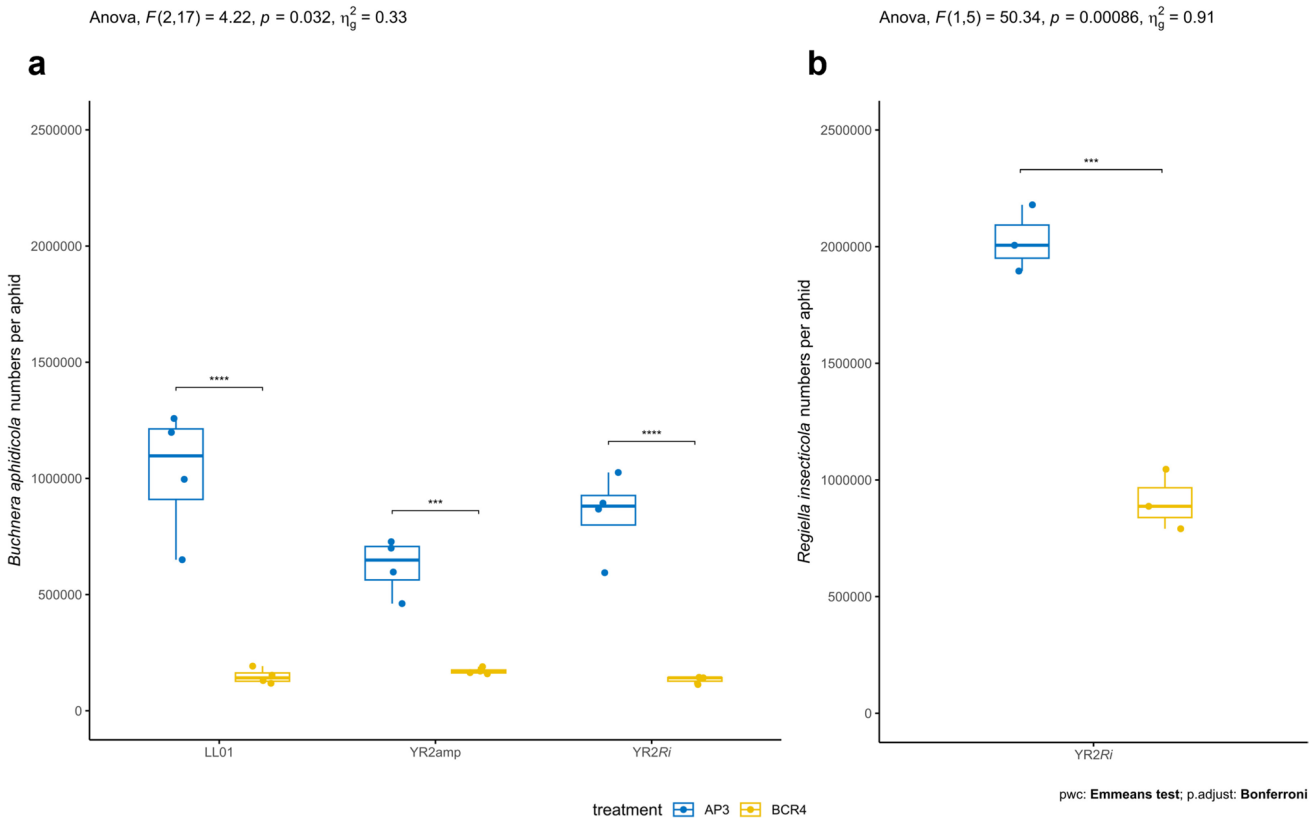

Fig. 6 Effect of BCR4 ingestion on *Buchnera aphidicola* and *Regiella insecticola* numbers in three *Acyrthosiphon pisum* lines. Number of bacteria per aphid fed on AP3 (blue) or in AP3 + BCR4 (1000 µg/ml) (yellow) for **a** *B. aphidicola* from different lines (*i.e.* LL01, YR2-amp, and YR2-*Ri*) and **b** the *R. insecticola* from YR2-*Ri* line. (pwc = pairwise comparison with emmeans test: ****: $p < 0.0001$; ***: $p < 0.001$. $n = 4$ per aphid, per line and per treatment)

bacteriocyte morphology and reduction of bacteriocyte numbers after treatment with indolicidin. Our study shows that BCR4 does not destroy the bacteriocyte membrane at the tested doses. Moreover, no disruption of the digestive tract membranes was observed. Nevertheless, an ultrastructural alteration of the membranes cannot be excluded. Further studies are needed to dissect the precise effect of BCR4 on aphid cell membranes using appropriate approaches such as transmission electron microscopy.

With FISH, we also show that the *R. insecticola* signal appears to be less intense around the bacteriocytes than in the rest of the body.

Taken together, the disturbance in the anatomical organization of bacteriocytes and the reduction of *R. insecticola* signal suggests an effect of BCR4 on both symbiotic populations. To test this hypothesis, we used flow cytometry to get insights into the number of symbiotic bacteria. Comparison between symbiont numbers in YR2-*Ri* lines showed that *R. insecticola* is more than twice as abundant as *B. aphidicola* in both control and treated aphids. This is consistent with a previous study conducted on another parthenogenetic line (LSR1) of *A. pisum* (Goldstein et al. 2023) showing similar relative abundance for certain *R. insecticola* strains. Concerning the treatment with BCR4, flow cytometry showed a massive reduction (72–84%) of *B. aphidicola* numbers in BCR4-treated aphids. This method also showed that BCR4 has a strong, but lower effect (58%) on *R. insecticola* numbers. Comparable results have been obtained by Luna-Ramirez et al. (2017), who showed that ingestion of scorpion AMPs reduces the symbiotic load (*B. aphidicola* and *Serratia symbiotica)* in *A. pisum*, and argue that insecticidal effects of those AMPs are due to a direct effect on symbionts. Importantly, previous studies have shown that BCR4 is bactericidal in vitro against *E. coli*, a Gram-negative bacterium closely related to *B. aphidicola* (Uchi et al. 2019; Loth et al. 2022). Combined with these observations, our results suggest that orally administered BCR4 defensin could have an in vivo bactericidal effect on aphid symbionts in three *A. pisum* parthenogenetic lines, thereby having a negative, indirect impact on their growth, survival, and embryo development.

Interestingly, *B. aphidicola* symbionts appear to be more sensitive to BCR4 than *R. insecticola*. Defensins typically target bacterial membranes, often leading to membrane

disruption or pore formation (Cociancich et al. 1993; Shai 2002; Wimley 2010; Wu et al. 2018; Agadi et al. 2022) and it has been shown that BCR4 alter *E. coli* morphology and membrane permeability (Uchi et al. 2019). It has been demonstrated that the *B. aphidicola* outer membrane lacks lipopolysaccharides (LPS) (Shigenobu et al. 2000; Charles et al. 2011), which may explain its greater vulnerability compared to *R. insecticola*. As with other AMPs, one could propose that BCR4 exhibits a selective activity, being more specific against one symbiont (here *B. aphidicola*) than another (here *R. insecticola*). For instance, in the bean bug *Riptortus pedestris*, several AMPs called Crypt-specific Cysteine-Rich peptides (CCRs) are expressed in the specialized posterior midgut region M4, prior to the acquisition of orally transmitted microbiota. This results in a selective barrier against unwanted bacteria, which facilitates the colonization by its beneficial symbiont *Caballeronia insecticola* (formerly known as *Burkholderia insecticola*) that is resistant to host CCRs (Lachat et al. 2024). Once established, beneficial *C. insecticola* becomes vulnerable to host AMPs due to an alteration in its LPS, which allows the host to regulate its gut symbionts (Kim et al. 2015). Similarly, in *Drosophila*, AMPs expressed in the gut have been shown to exhibit high selectivity depending on the invading pathogen (Hanson et al. 2019). As an example, the commensal, *Lactiplantibacillus plantarum*, is resistant to host AMPs during infection phases, allowing it to stably colonize the fly gut (Arias-Rojas et al. 2023).

Further investigations are needed to explore the potential of orally administered BCRs in selectively targeting different facultative symbionts in *A. pisum*, and more generally in aphids.

Conclusion

In conclusion, this study highlights the detrimental effect of BCR4 on aphids when administered orally. Further research is needed to understand the functions of the BCR family in aphids and their potential use as bioinsecticides. Targeting insect symbionts in the context of pest management is increasingly being discussed (Arora & Douglas 2017; Gonella et al. 2020; Noman et al. 2020; Sinno et al. 2020; Gonella & Alma 2023; Rupawate et al. 2023). Combined with advances in plant bioengineering (Bisht et al. 2019; Suhag et al. 2021; Mateos Fernández et al. 2022; Komal et al. 2023), and because they are restricted to the aphid lineage (Loth et al. 2022), BCR delivery could be a powerful tool to specifically control aphid pest through interference with their symbiont populations.

Author contributions

M.R.L., F.C. and P.D.S. conceived and designed research. C.J. and G.D. prepared the AP3 medium. C.S. and V.A. produced the BCR4 peptide. H.T., I.R., and C.J. conducted bioassays experiments. H.T. and S.B. performed the survival and mass monitoring and analyzed the data. H.T., K.G. and G.L. conducted histological preparations and observations. H.T., K.G. and G.L. conducted dissections and macroscopical observations. K.G. and G.L. performed flow cytometry experiments and K.G. analyzed the data. J.C.S. provided both YR2 *A. pisum* lines. H.T. prepared the figures and wrote the first draft F.R., S.B., J.C.S., M.R.L., F.C. and P.D.S. reviewed and corrected the draft. All authors read and approved the manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval No international, national, and institutional guidelines for the care and use of animals were needed and followed.

References

Agadi N, Maity A, Jha AK et al (2022) Distinct mode of membrane interaction and disintegration by diverse class of antimicrobial peptides. Biochimica Et Biophysica Acta (BBA)—Biomembranes 1864:184047. https://doi.org/10.1016/j.bbamem.2022.184047

Almeida CV, de Oliveira CFR, dos Santos EL et al (2021) Differential interactions of the antimicrobial peptide, RQ18, with phospholipids and cholesterol modulate its selectivity for microorganism membranes. Biochimica Et Biophys Acta (BBA)–gen Subj 1865:129937. https://doi.org/10.1016/j.bbagen.2021.129937

Andersson DI, Hughes D, Kubicek-Sutherland JZ (2016) Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug Resist Updates 26:43–57. https://doi.org/10.1016/j.drup.2016.04.002

Arias-Rojas A, Frahm D, Hurwitz R, et al (2023) Resistance to host antimicrobial peptides mediates resilience of gut commensals during infection and aging in *Drosophila*. Proceedings of the National

Academy of Sciences 120. https://doi.org/10.1073/pnas.2305649120
Arora AK, Douglas AE (2017) Hype or opportunity? Using microbial symbionts in novel strategies for insect pest control. J Insect Physiol 103:10–17. https://doi.org/10.1016/j.jinsphys.2017.09.011
Barassé V, Jouvensal L, Boy G et al (2023) Discovery of an insect neuroactive helix ring peptide from ant venom. Toxins 15:600. https://doi.org/10.3390/toxins15100600
Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59:155–189. https://doi.org/10.1146/annurev.micro.59.030804.121041
Benhamou S, Rahioui I, Henri H, et al (2021) Cytotype affects the capability of the whitefly *Bemisia tabaci* MED species to feed and oviposit on an unfavorable host plant. mBio 12:e0073021. https://doi.org/10.1128/mBio.00730-21
Bisht DS, Bhatia V, Bhattacharya R (2019) Improving plant-resistance to insect-pests and pathogens: the new opportunities through targeted genome editing. Semin Cell Dev Biol 96:65–76. https://doi.org/10.1016/j.semcdb.2019.04.008
Braendle C, Miura T, Bickel R, et al (2003) Developmental origin and evolution of bacteriocytes in the aphid–*Buchnera* symbiosis. PLOS Biology 1. https://doi.org/10.1371/journal.pbio.0000021
Calatayud P-A (2000) Influence of linamarin and rutin on biological performances of *Phenacoccus manihoti* in artificial diets. Entomol Exp Appl 96:81–86. https://doi.org/10.1046/j.1570-7458.2000.00681.x
Calevro F, Tagu D, Callaerts P (2019) Acyrthosiphon pisum. Trends Genet 35:781–782. https://doi.org/10.1016/j.tig.2019.07.003
Charles H, Balmand S, Lamelas A et al (2011) A genomic reappraisal of symbiotic function in the aphid/*Buchnera* symbiosis: reduced transporter sets and variable membrane organisations. PLoS ONE. https://doi.org/10.1371/journal.pone.0029096
Cociancich S, Ghazi A, Hetru C et al (1993) Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. J Biol Chem 268:19239–19245. https://doi.org/10.1016/S0021-9258(19)36505-6
Darby AC, Douglas AE (2003) Elucidation of the transmission patterns of an insect-borne bacterium. Appl Environ Microbiol 69:4403–4407. https://doi.org/10.1128/AEM.69.8.4403-4407.2003
Dedryver C-A, Le Ralec A, Fabre F (2010) The conflicting relationships between aphids and men: a review of aphid damage and control strategies. CR Biol 333:539–553. https://doi.org/10.1016/j.crvi.2010.03.009
El Fakhouri K, Sabraoui A, Kehel Z, El Bouhssini M (2021) Population dynamics and yield loss assessment for pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), on lentil in Morocco. InSects 12:1080. https://doi.org/10.3390/insects12121080
Febvay G, Delobel B, Rahbé Y (1988) Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera: Aphididae). Can J Zool 66:2449–2453. https://doi.org/10.1139/z88-362
Ferrari J, Scarborough CL, Godfray HCJ (2007) Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. Oecologia 153:323–329. https://doi.org/10.1007/s00442-007-0730-2
Frago E, Mala M, Weldegergis BT et al (2017) Symbionts protect aphids from parasitic wasps by attenuating herbivore-induced plant volatiles. Nat Commun 8:1860. https://doi.org/10.1038/s41467-017-01935-0
Goldstein EB, de Anda AY, Henry LM, Parker BJ (2023) Variation in density, immune gene suppression, and coinfection outcomes among strains of the aphid endosymbiont *Regiella insecticola*. Evolution 77:1704–1711. https://doi.org/10.1093/evolut/qpad071
Gonella E, Alma A (2023) The role of symbiont-targeted strategies in the management of Pentatomidae and Tephritidae pests under an integrated vision. Agronomy 13:868. https://doi.org/10.3390/agronomy13030868
Gonella E, Orrù B, Marasco R et al (2020) Disruption of host-symbiont associations for the symbiotic control and management of pentatomid agricultural pests—a review. Front Microbiol 11:547031. https://doi.org/10.3389/fmicb.2020.547031
Goulson D, 232 signatories (2018) Call to restrict neonicotinoids. Science 360:973–973. https://doi.org/10.1126/science.aau0432
Greco I, Molchanova N, Holmedal E et al (2020) Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. Sci Rep 10:13206. https://doi.org/10.1038/s41598-020-69995-9
Gula LT (2023) Researchers helping protect crops from pests. NIFA. https://www.nifa.usda.gov/about-nifa/blogs/researchers-helping-protect-crops-pests. Accessed 26 Jun 2024
Guo J, Hatt S, He K et al (2017) Nine facultative endosymbionts in aphids. A Rev J Asia-Pac Entomol 20:794–801. https://doi.org/10.1016/j.aspen.2017.03.025
Hansen AK, Moran NA (2014) The impact of microbial symbionts on host plant utilization by herbivorous insects. Mol Ecol 23:1473–1496. https://doi.org/10.1111/mec.12421
Hanson MA, Dostálová A, Ceroni C et al (2019) Synergy and remarkable specificity of antimicrobial peptides *in vivo* using a systematic knockout approach. Elife. https://doi.org/10.7554/eLife.44341
Higashi CHV, Nichols WL, Chevignon G et al (2023) An aphid symbiont confers protection against a specialized RNA virus, another increases vulnerability to the same pathogen. Mol Ecol 32:936–950. https://doi.org/10.1111/mec.16801
Hlina BL, Birceanu O, Robinson CS et al (2021) The relationship between thermal physiology and lampricide sensitivity in larval sea lamprey (*Petromyzon marinus*). J Gt Lakes Res 47:S284. https://doi.org/10.1016/j.jglr.2021.10.002
Ho TNT, Turner A, Pham SH et al (2023) Cysteine-rich peptides: from bioactivity to bioinsecticide applications. Toxicon 230:107173. https://doi.org/10.1016/j.toxicon.2023.107173
Hogenhout SA, Ammar E-D, Whitfield AE, Redinbaugh MG (2008) Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol 46:327–359. https://doi.org/10.1146/annurev.phyto.022508.092135
Jahan H, Khudr MS, Arafeh A, Hager R (2023) Exposure to heat stress leads to striking clone-specific nymph deformity in pea aphid. PLOS ONE 18. https://doi.org/10.1371/journal.pone.0282449
Kim JK, Son DW, Kim C-H et al (2015) Insect gut symbiont susceptibility to host antimicrobial peptides caused by alteration of the bacterial cell envelope. J Biol Chem 290:21042–21053. https://doi.org/10.1074/jbc.M115.651158
Koga R, Tsuchida T, Sakurai M, Fukatsu T (2007) Selective elimination of aphid endosymbionts: effects of antibiotic dose and host genotype, and fitness consequences. FEMS Microbiol Ecol 60:229–239. https://doi.org/10.1111/j.1574-6941.2007.00284.x
Koga R, Meng X-Y, Tsuchida T, Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. Proc Natl Acad Sci 109:E1230–E1237. https://doi.org/10.1073/pnas.1119212109
Komal J, Desai HR, Samal I et al (2023) Unveiling the genetic symphony: harnessing CRISPR-Cas genome editing for effective insect pest management. Plants 12:3961. https://doi.org/10.3390/plants12233961
Kontsedalov S, Zchori-Fein E, Chiel E et al (2008) The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. Pest Manag Sci 64:789–792. https://doi.org/10.1002/ps.1595
Lachat J, Lextrait G, Jouan R et al (2024) Hundreds of antimicrobial peptides create a selective barrier for insect gut symbionts. Proc Natl Acad Sci 121:71. https://doi.org/10.1073/pnas.2401802121

Le-Feuvre RR, Ramírez CC, Olea N, Meza-Basso L (2007) Effect of the antimicrobial peptide indolicidin on the green peach aphid *Myzus persicae* (Sulzer). J Appl Entomol 131:71–75. https://doi.org/10.1111/j.1439-0418.2006.01117.x

Lemoine MM, Engl T, Kaltenpoth M (2020) Microbial symbionts expanding or constraining abiotic niche space in insects. Curr Opin Insect Sci 39:14–20. https://doi.org/10.1016/j.cois.2020.01.003

Li Q, Fan J, Sun J et al (2018) Plant-mediated horizontal transmission of *Hamiltonella defensa* in the wheat aphid *Sitobion miscanthi*. J Agric Food Chem 66:13367–13377. https://doi.org/10.1021/acs.jafc.8b04828

Li Q, Sun J, Qin Y et al (2021) Reduced insecticide susceptibility of the wheat aphid after infection by the secondary bacterial symbiont *Hamiltonella defensa*. Pest Manag Sci 77:1936–1944. https://doi.org/10.1002/ps.6221

Libbrecht R, Gwynn DM, Fellowes MDE (2007) *Aphidius ervi* preferentially attacks the green morph of the pea aphid, *Acyrthosiphon pisum*. J Insect Behav 20:25–32. https://doi.org/10.1007/s10905-006-9055-y

Loth K, Parisot N, Paquet F et al (2022) Aphid BCR4 structure and activity uncover a new defensin peptide superfamily. Int J Mol Sci 23:12480. https://doi.org/10.3390/ijms232012480

Luna-Ramirez K, Skaljac M, Grotmann J et al (2017) Orally delivered scorpion antimicrobial peptides exhibit activity against pea aphid (*Acyrthosiphon pisum*) and its bacterial symbionts. Toxins 9:261. https://doi.org/10.3390/toxins9090261

Luo C, Belghazi M, Schmitz A et al (2021) Hosting certain facultative symbionts modulates the phenoloxidase activity and immune response of the pea aphid *Acyrthosiphon pisum*. Insect Sci 28:1780–1799. https://doi.org/10.1111/1744-7917.12888

Luo K, Zhao H, Wang X, Kang Z (2022) Prevalent pest management strategies for grain aphids: opportunities and challenges. Front Plant Sci. https://doi.org/10.3389/fpls.2021.790919

Man Y, Li D, Wang M et al (2023) Indirect and direct interactions between grain aphid and parasitoid in the presence of symbiont *Regiella insecticola*. CABI Agric Biosci 4:59. https://doi.org/10.1186/s43170-023-00202-1

Martinez AJ, Doremus MR, Kraft LJ et al (2018) Multi-modal defences in aphids offer redundant protection and increased costs likely impeding a protective mutualism. J Anim Ecol 87:464–477. https://doi.org/10.1111/1365-2656.12675

Mason AJ, Marquette A, Bechinger B (2007) Zwitterionic phospholipids and sterols modulate antimicrobial peptide-induced membrane destabilization. Biophys J 93:4289–4299. https://doi.org/10.1529/biophysj.107.116681

Mateos Fernández R, Petek M, Gerasymenko I et al (2022) Insect pest management in the age of synthetic biology. Plant Biotechnol J 20:25–36. https://doi.org/10.1111/pbi.13685

Miura T, Braendle C, Shingleton A et al (2003) A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). J Exp Zool B Mol Dev Evol 295B:59–81. https://doi.org/10.1002/jez.b.3

Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol Entomol 27:189–195. https://doi.org/10.1046/j.1365-2311.2002.00393.x

Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Appl Environ Microbiol 71:3302–3310. https://doi.org/10.1128/AEM.71.6.3302-3310.2005

Noman MS, Liu L, Bai Z, Li Z (2020) Tephritidae bacterial symbionts: potentials for pest management. Bull Entomol Res 110:1–14. https://doi.org/10.1017/S0007485319000403

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci 100:1803–1807. https://doi.org/10.1073/pnas.0335320100

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55:247–266. https://doi.org/10.1146/annurev-ento-112408-085305

Parker BJ, Spragg CJ, Altincicek B, Gerardo NM (2013) Symbiont-mediated protection against fungal pathogens in pea aphids: A role for pathogen specificity? Appl Environ Microbiol 79:2455–2458. https://doi.org/10.1128/AEM.03193-12

Parker BJ, Hrček J, McLean AHC, Godfray HCJ (2017) Genotype specificity among hosts, pathogens, and beneficial microbes influences the strength of symbiont-mediated protection. Evolution 71:1222–1231. https://doi.org/10.1111/evo.13216

Peccoud J, Bonhomme J, Mahéo F et al (2014) Inheritance patterns of secondary symbionts during sexual reproduction of pea aphid biotypes. Insect Science 21:291–300. https://doi.org/10.1111/1744-7917.12083

Pons I, Renoz F, Noël C, Hance T (2019a) Circulation of the cultivable symbiont *Serratia symbiotica* in aphids is mediated by plants. Front Microbiol. https://doi.org/10.3389/fmicb.2019.00764

Pons I, Renoz F, Noël C, Hance T (2019b) New insights into the nature of symbiotic associations in aphids: infection process, biological effects, and transmission mode of cultivable *Serratia symbiotica* bacteria. Appl Environ Microbiol. https://doi.org/10.1128/AEM.02445-18

R Core Team (2023) R: A language and environment for statistical computing. R foundation for statistical computing, Vienna. https://www.R-project.org/

Rabbinge R, Drees EM, van der Graaf M et al (1981) Damage effects of cereal aphids in wheat. Neth J Plant Pathol 87:217–232. https://doi.org/10.1007/BF02084437

Rahbé Y, Febvay G (1993) Protein toxicity to aphids: an in vitro test on *Acyrthosiphon pisum*. Entomol Exp Appl 67:149–160. https://doi.org/10.1111/j.1570-7458.1993.tb01663.x

Rahbé Y, Febvay G, Delobel B, Bournoville R (1988) *Acyrthosiphon pisum* performance in response to the sugar and amino acid composition of artificial diets, and its relation to lucerne varietal resistance. Entomol Exp Appl 48:283–292. https://doi.org/10.1111/j.1570-7458.1988.tb01175.x

Renoz F (2024) The nutritional dimension of facultative bacterial symbiosis in aphids: current status and methodological considerations for future research. Curr Res Insect Sci 5:100070. https://doi.org/10.1016/j.cris.2023.100070

Renoz F, Pons I, Vanderpoorten A et al (2019) Evidence for gut-associated *Serratia symbiotica* in wild aphids and ants provides new perspectives on the evolution of bacterial mutualism in insects. Microb Ecol 78:159–169. https://doi.org/10.1007/s00248-018-1265-2

Ribeiro Lopes M, Simonet P, Duport G et al (2021) Isolation of insect bacteriocytes as a platform for transcriptomic analyses. Methods Mol Biol 2170:185–198. https://doi.org/10.1007/978-1-0716-0743-5_13

Ribeiro Lopes M, Gaget K, Renoz F et al (2022) Bacteriocyte plasticity in pea aphids facing amino acid stress or starvation during development. Front Physiol. https://doi.org/10.3389/fphys.2022.982920

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLoS ONE. https://doi.org/10.1371/journal.pone.0146021

Rupawate PS, Roylawar P, Khandagale K et al (2023) Role of gut symbionts of insect pests: a novel target for insect-pest control. Front Microbiol. https://doi.org/10.3389/fmicb.2023.1146390

Russell JA, Moran NA (2005) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc Royal Soc b: Biol Sci 273:603–610. https://doi.org/10.1098/rspb.2005.3348

Sapountzis P, Duport G, Balmand S et al (2014) New insight into the RNA interference response against cathepsin-L gene in the pea aphid, *Acyrthosiphon pisum*: molting or gut phenotypes specifically induced by injection or feeding treatments. Insect Biochem Mol Biol 51:20–32. https://doi.org/10.1016/j.ibmb.2014.05.005

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. Science 310:1781–1781. https://doi.org/10.1126/science.1120180

Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Pept Sci 66:236–248. https://doi.org/10.1002/bip.10260

Shigenobu S, Stern DL (2013) Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. Proc Royal Soc B: Biol Sci 280:20121952. https://doi.org/10.1098/rspb.2012.1952

Shigenobu S, Watanabe H, Hattori M et al (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS Nat 407:81–86. https://doi.org/10.1038/35024074

Simon J-C, Boutin S, Tsuchida T et al (2011) Facultative symbiont infections affect aphid reproduction. PLoS ONE. https://doi.org/10.1371/journal.pone.0021831

Simonet P, Duport G, Gaget K et al (2016) Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis. Sci Rep 6:19967. https://doi.org/10.1038/srep19967

Simonet P, Gaget K, Balmand S et al (2018) Bacteriocyte cell death in the pea aphid/*Buchnera* symbiotic system. Proc Natl Acad Sci 115:E1819–E1828. https://doi.org/10.1073/pnas.1720237115

Sinno M, Bézier A, Vinale F et al (2020) Symbiosis disruption in the olive fruit fly, *Bactrocera oleae* (Rossi), as a potential tool for sustainable control. Pest Manag Sci 76:3199–3207. https://doi.org/10.1002/ps.5875

Skaljac M, Kirfel P, Grotmann J, Vilcinskas A (2018) Fitness costs of infection with *Serratia symbiotica* are associated with greater susceptibility to insecticides in the pea aphid. Pest Manag Sci 74:1829–1836. https://doi.org/10.1002/ps.4881

Smith TE, Moran NA (2020) Coordination of host and symbiont gene expression reveals a metabolic tug-of-war between aphids and *Buchnera*. Proc Natl Acad Sci 117:2113–2121. https://doi.org/10.1073/pnas.1916748117

Sochard C, Le Floch M, Anton S et al (2021) Limited influence of gain and loss of symbionts on host plant selection in specialized pea aphid genotypes. Entomol Generalis. https://doi.org/10.1127/entomologia/2020/1076

Suhag A, Yadav H, Chaudhary D et al (2021) Biotechnological interventions for the sustainable management of a global pest, whitefly (*Bemisia tabaci*). Insect Sci 28:1228–1252. https://doi.org/10.1111/1744-7917.12853

Therneau T (2024). *A Package for Survival Analysis in R.* R package version 3.7–0, https://CRAN.R-project.org/package=survival

Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. Science 303:1989–1989. https://doi.org/10.1126/science.1094611

Tsuchida T, Koga R, Meng XY et al (2005) Characterization of a facultative endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum*. Microb Ecol 49:126–133. https://doi.org/10.1007/s00248-004-0216-2

Tsuchida T, Koga R, Fujiwara A, Fukatsu T (2014) Phenotypic effect of "*Candidatus Rickettsiella viridis*", a facultative symbiont of the pea aphid (*Acyrthosiphon pisum*), and its interaction with a coexisting symbiont. Appl Environ Microbiol 80:525. https://doi.org/10.1128/AEM.03049-13

Uchi N, Fukudome M, Nozaki N et al (2019) Antimicrobial activities of cysteine-rich peptides specific to bacteriocytes of the pea aphid *Acyrthosiphon pisum*. Microbes Environ 34:155–160. https://doi.org/10.1264/jsme2.ME18148

Vaucher RA, Teixeira ML, Brandelli A (2010) Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells. Curr Microbiol 60:1–5. https://doi.org/10.1007/s00284-009-9490-z

Wernegreen JJ (2012) Endosymbiosis. Curr Biol 22:R555–R561. https://doi.org/10.1016/j.cub.2012.06.010

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5:905–917. https://doi.org/10.1021/cb1001558

Wu Q, Patočka J, Kuča K (2018) Insect antimicrobial peptides, a mini review. Toxins 10:461. https://doi.org/10.3390/toxins10110461

Zytynska SE, Tighiouart K, Frago E (2021) Benefits and costs of hosting facultative symbionts in plant-sucking insects: a meta-analysis. Mol Ecol 30:2483–2494. https://doi.org/10.1111/mec.15897