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ORIGINAL ARTICLE

The clip cage conundrum: Assessing the interplay of confinement method and aphid genotype in fitness studies

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Abstract Behavior and fitness are important ecological traits frequently measured in insect bioassays. A common method to measure them in soft-bodied herbivorous insects involves confining individuals to plant leaves using clip cages. Although studies have previously highlighted the negative effects of clip cages on leaf physiology, little is known about the impact that using this confinement method has on insect fitness. The responses of different aphid genotypes/clones to different containment methods have not previously been investigated. Here we measured key fitness traits (intrinsic rate of natural increase, mean relative growth rate, time to reach reproductive adulthood and population doubling time) in the potato aphid, *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae), when confined to plants using two methods: (1) clip cages to confine aphids to individual strawberry leaves and (2) a mesh bag to confine aphids to whole strawberry plants. Our study identified a strong negative impact on all the measured aphid fitness traits when using clip cages instead of mesh bags. We also identified genotype-specific differences in response to confinement method, where clip cage confinement differentially affected the fitness of a given aphid genotype compared to the same genotype on whole plants. These results suggest that clip cage use should be carefully considered when experiments seek to quantify insect fitness and that whole plants should be used wherever possible. Given the prevalence of clip cage use in insect bioassays, our results highlight the need for caution when interpreting the existing literature as confinement method significantly impacts aphid fitness depending on their genotype.

Key words aphid clonal variation; aphid fitness; clip cages; confinement method; feeding site; insect bioassays

Introduction

Measuring behavior and fitness traits is core to many natural history and ecological studies. For example, in the case of economically important insect herbivore species, both behavior and fitness can have a significant impact on pest management decisions in crop production systems (van Emden & Harrington, 2017). Individual fitness traits such as development, reproduction (fecundity), and survival are often used in an ecological context to understand host range, plant–herbivore interactions, and evolution. Those same traits can also be used to determine the likely economic impact of an insect herbivore on different crops or crop genotypes and to evaluate plant resistance to an insect pest (Lamb *et al.*, 2009).

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Herbivorous insects and their host plants have a relationship driven by co-evolution. Damage inflicted by insect feeding is a strong selection pressure for plants to develop defensive strategies such as physical traits (e.g., trichomes) and secondary metabolites. In response, insects have evolved counteradaptations to plant defensive traits that they are typically subjected to by developing morphological, biochemical, and behavioral traits (War *et al.*, 2018). Therefore, insect fitness can be influenced by the defensive adaptations of their host and their own adaptative responses (Zvereva & Kozlov, 2016). This results in an array of physical and chemical defenses in both organisms that influence the ability of an insect herbivore to infest and feed on a host plant (Caillaud & Niemeyer, 1996; Mehrparvar *et al.*, 2019). For example, preference for cowpea and cotton plant hosts has been noted for the cowpea aphid *Aphis craccivora* Koch in comparison with other potential host plants and is negatively correlated with plant trichome density, phenol content, and level of carbohydrates (Routray *et al.*, 2020). Host plant preference caused by insect counteradaptations to plant defenses can lead to the evolution of insect biotypes that specialize on a small number of closely related plant species. In the pea aphid (*Acyrthosiphon pisum* Harris), at least 15 biotypes have been described (Birkle & Douglas, 1999), which exhibit different salivary effector gene expression levels that are thought to be required for host plant adaptation (Boulain *et al.*, 2019). Insect species might also have a preferred feeding site on a specific host plant species, (stems, flowers, abaxial leaf surface, adaxial leaf surface, and roots) due to plant structure-specific nutritional composition or defensive adaptations (Vilcinskas, 2016; Mehrparvar *et al.*, 2019). In the case of phloem-feeding aphids, feeding site appears to be driven by nutritional composition rather than physical trait differences (Douglas, 2008; Nalam *et al.*, 2021). Plant structure preferences of different aphid species are, therefore, frequently reported. For example, the lettuce aphid (*Nasonovia ribisnigri* Mosley) prefers inner lettuce leaves but the potato aphid (*Macrosiphum euphorbiae* Thomas) prefers outer leaves (Shrestha *et al.*, 2017). However, it is unknown if there is within-species variation in the preferred feeding sites of aphids in many cases.

Quantifying fitness traits can be complicated for herbivores like aphids where the same individual or group must be isolated and followed over time to gather life history information. Clip cages are one approach used to confine small insects in a specific location on host plant leaves and exclude other individuals and natural enemies (Taravati & Mannion, 2016). For this reason, clip cages have become a standard method for entomologists to study the fitness of soft-body insects like aphids under laboratory conditions (Vilcinskas, 2016). A range of clip cage designs have been developed by changing their size or weight and by modifying their shape to suit different host plants (Lamb *et al.*, 2009). However, not only do clip cages restrict where aphids can feed, it has been demonstrated that clip cages, no matter which configuration is used, can have unexpected effects on the leaf such as permanent physical damage, changes in physiology, and negative effects on photosynthesis (Crafts-Brandner & Chu, 1999; Moore *et al.*, 2003; Haas *et al.*, 2018), all of which can have spatial and temporal effects on the feeding aphid. These types of physiological changes might influence the outcome of insect fitness measurements. For example, plant physical disturbance can trigger the release of leaf volatile compounds in maize that deter the bird cherry-oat aphid (*Rhopalosiphum padi* L.) from selecting the plants as a host (Markovic *et al.*, 2014), something that can negatively impact on aphid fitness in no-choice experiments. In addition, fitness traits (developmental time, fecundity, and adult weight) of the grain aphid (*Sitobion avenae* Fabricius) in response to plant stress have been shown to vary between genetically distinct clonal lines (Liu *et al.*, 2018). Therefore, clip cages might indirectly impact insect herbivore fitness through their effects on leaf traits and physiological stress (Moore *et al.*, 2003) in addition to the direct effect of restricting feeding site choice. Another experimental method to measure insect fitness, which can be used instead of clip cages, involves using entire plants enclosed in small cages to isolate the herbivores (Mowry, 1993). Use of whole plant techniques, in addition to having fewer direct effects on plants, also gives insects access to different parts of the host and with that the freedom to select the site in which to reach their maximum fitness without constraints. However, this method is both time and space consuming, which often limits the number of replicates or observation frequency. In addition, covering plants with meshed bags can also impact plant physiology by reducing the amount of light that the plant receives, which can affect plant tissue nutrient and secondary metabolite concentrations (Stamp & Bowers, 1994), or by stressing the plants due to mechanical overstimulation (Coutand, 2020). Although it has not been explored, it could also have undesirable effects on aphids, by for example, increasing disturbance.

Potato aphids are one of the most economically important pests of solanaceous and horticultural crops (Saguez *et al.*, 2013). This aphid species can cause direct feeding damage to its host plant leaves, stems, and fruits as well as indirect damage from necrotic spots and fungal growth on the leaves (sooty mold) from honeydew deposition (van Emden & Harrington, 2017). The potato

aphid is also a vector of viruses in *Solanaceae* plants, such as potato virus Y, potato leafroll virus and beet yellow virus (Taylor, 2013). However, no virus transmission has been described for other hosts, such as strawberries. Although this aphid species has a broad host range, studies on potato aphid preference for different host plants are scarce. However, it has been reported that this species prefers the weed *Solanum sarrachoides* Sendtner (Solanaceae) over potato plants *Solanum tuberosum* L. (Solanaceae) (Srinivasan, 2011). It has also been shown that even if different potato aphid clones attack the same crop species, the potential to overcome plant defenses varies depending on aphid genotype and therefore their fitness can vary on the same host (Karley *et al.*, 2017). In general, the role of genetic variation on insect fitness has been associated with phenotypic variation in terms of size (which has been linked to fecundity and longevity), physiological defenses against plant compounds, physiological defenses against parasitoids, and behavioral differences (Clarke, 2013; Beukeboom, 2018; Humphreys *et al.*, 2021b), all of which could impact aphid fitness depending on the environment. In addition, aphid facultative symbionts are known to influence aphid fitness in some aphid species. For example, in the corn leaf aphid *Rhopalosiphum maidis* Fitch, the presence of the symbiont *Hamiltonella defensa* results in shorter development times, reduced aphid survival rate, increased fecundity, and extended longevity, while the presence of the symbiont *Regiella insecticola* results in longer development times and lower adult weights (Liu *et al.*, 2023). In the pea aphid, individuals with *Rickettsia* sp. showed reduced longevity, and those infected with *Spiroplasma* or *H. defensa* showed reduced fecundity (Simon *et al.*, 2011), while *Aphis fabae* Scopoli infected with *H. defensa* reduced the longevity and fecundity of unparasitized aphids (Cayetano *et al.*, 2015). Nevertheless, no effects of secondary endosymbiont infections have been described for potato aphid fitness to date (Clarke, 2013).

There have been many fitness studies carried out on potato aphid fitness using clip cages, especially to determine host plant resistance (Pompon *et al.*, 2011; Karley *et al.*, 2017; Beetge & Krüger, 2019). Nevertheless, there is no information on the effect of the method used to quantify potato aphid fitness nor, for any other aphid species. Similarly, there is no information on whether there are differential genotypic/clonal responses to aphid containment method. Thus, the present study aims to investigate if (1) containment method impacts aphid fitness and (2) there is an interaction between containment method and aphid clonal variation in aphid fitness. To this end, we investigated potato aphid fitness traits when they can move freely on strawberry plants and when they are confined to the leaves of strawberry plants using clip cages.

Materials and methods

Plants

Strawberries (*Fragaria* × *ananassa* variety "Elsanta") were exclusively used throughout this study. Prior to their use, all plants were stored as dormant bare-root strawberry plants. These were then planted in plastic pots (12.6 cm diameter, Pöppleman Plastics UK Ltd, Hull, UK) containing compost (John Innes n°02, Arthur Bowers, Westland Horticulture Limited, Cheshire, UK) and placed in an insect proof mesh cage (60 cm × 60 cm × 60 cm, BugDorm-6S610, MegaView Science Co. Ltd, Taichung, Taiwan, China) under natural light conditions at approximately 20 °C within a glasshouse to be grown on for aphid culturing or use in the experiment.

Aphid cultures and age standardized cohorts

Stock clonal cultures of potato aphids used in this experiment originated from a single individual from four populations collected at different times and/or locations (Table 1). These cultures were each reared on strawberry plants (grown as previously described) in an insect proof mesh cage (47.5 cm × 47.5 cm × 47.5 cm, BugDorm-4S4545, MegaView Science Co. Ltd, Taichung, Taiwan, China) within a controlled environmental room maintained at 18 °C and 60% relative humidity with a 16 h : 8 h photoperiod. Each culture was maintained by transferring aphids onto small strawberry plants (Growth stage 13 of the BBCH-scale for strawberry), with at least three fully unfolded trifoliate leaves, weekly throughout the study period.

To generate age standardized cohorts of apterous aphids from each clone, 20 apterous adult aphids were transferred onto an individual strawberry plant placed within an insect proof mesh cage (47.5 cm × 47.5 cm × 47.5 cm, BugDorm-4S4545) using a size 000 paintbrush. After 24 h, adult aphids were removed from the plants using a size 000 paintbrush to leave only first instar nymphs. These were subsequently left to develop into adults.

Characterizing potato aphid clones

Genomic DNA was extracted from whole potato aphids from the four clonal lines listed in Table 1 using

Table 1 Collection date and location of the founding aphids for the four *M. euphorbiae* clonal lines used in the experiment. All collected aphids originated from strawberry plants (*Fragaria* × *anannassa*).

Potato aphid (*M. euphorbiae*) clonal line	Collection date	Location	Morphotype color
HAU_01	2018	Kent, UK	Green
HAU_02	2021	Woore, Staffordshire, UK	Green
HAU_03	2021	NIAB East Malling, Kent, UK	Green
HAU_04	2021	NIAB East Malling, Kent, UK	Pink

the DNeasy® Blood and Tissue Kit (Qiagen, Crawley, UK), following the manufacturer's protocol for extracting DNA from insects with a micropestle. The concentration and purity of each aphid DNA sample was analyzed spectrophotometrically using a NanoDrop ND-1000 Full spectrum UV-Visible spectrophotometer (ThermoFisher Scientific, Epsom, UK). With extracted DNA concentrations in the range 30–60 ng/μL, each DNA sample was diluted to reach a final concentration of 5 ng/μL and stored at −20 °C.

The four potato aphid clones were genotyped based on length polymorphisms for seven microsatellite loci, which have been used in previous research to differentiate clones of this aphid species (Clarke, 2013; Whitehead, 2019). Microsatellite primers (Table S1) were tagged with dyes on the 5 end (FAM, VIC, NED) and were used in 25 μL reactions using PCR beads (200 μmol/L of each dNTP (in 10 mmol/L Tris-HCL 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$), 2.5 U puReTaq DNA polymerase, 0.5 mmol/L of each primer, and 1 μL DNA template (5 ng/μL) (illustra puReTaq Ready-To-Go PCR Beads, Cytiva Life Sciences, UK). Products were separated by capillary electrophoresis using an ABI 3730 DNA Analyser (Life Technologies, Paisley, UK) with fragment sizes determined against GeneScan 500 LIZ internal lane size standards using ThermoFisher Scientific Peak Scanner Software v1.0.

Universal and specific primers for the 16S rRNA gene (Table S2) were used in a nested PCR to screen each of the four potato aphid clones for facultative bacterial endosymbionts. The universal 16S gene primers were first used to confirm the extraction of prokaryote DNA from the aphid samples using the aphid DNA as a template. The amplicon of the first reaction was diluted 1 : 20 for use as a template for the second PCR reaction. For the latter, all specific 16S rRNA primers for known secondary endosymbionts of aphids (*Hamiltonella defensa*, *Serratia symbiotica*, *Regiella insecticola*, *Fukatsuia symbiotica*, *Spiroplasma*, *Rickettsia* sp., *Ricketsiella* sp.) (Table S2) were used to determine the presence of the specific bacterial species. All PCR reactions were conducted in 25 μL volumes using PCR beads (200 μmol/L of each dNTP (in 10 mmol/L Tris-HCL 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$), 2.5 U puReTaq DNA polymerase, 0.5 mmol/L of each primer, and 1 μL DNA template (5 ng/μL) (illustra puReTaq Ready-To-Go PCR Beads, Cytiva Life Sciences, UK). The PCR products were run through 2% agarose gels (UltraPure™ agarose [Invitrogen/Life Technologies Ltd, Paisley, UK] in 1 × TBE buffer with GelRed® 10000X in water [Biotium Inc, California, USA]) to separate and estimate the size of the amplified DNA by comparing with a 50–2000 bp DNA molecular weight marker (EasyLadder I, Meridian Life Science, London, UK).

Potato aphid fitness bioassay

Strawberry plants were transferred to a controlled environmental room (Fitotron, Weiss Technik UK limited, Loughborough, UK) maintained at 60% relative humidity and 18 °C with a 16 : 8 h photoperiod (L : D) before use in fitness bioassays. Plants were watered twice weekly and no fertilizer was used before or during the experiment. The experiment was started when plants had at least one fully unfolded trifoliate leaf (Growth stage 11 of the BBCH-scale for strawberry), which was approximately 9 d after they were planted as bare-roots.

Individual aphid fitness was evaluated using two techniques for containing aphids onto plants: (1) mesh bags enclosing an aphid onto an entire plant (i.e., aphids can roam and select their feeding site, with potential effects of mesh bags on aphid disturbance) and (2) clip cages enclosing an aphid onto the abaxial surface of a single leaflet (i.e., aphids are confined to a single leaf for the experiment duration, with potential effects of clip cages on aphid disturbance and leaf physiology) (Fig. 1A). For technique (1), each plant was covered with a fine light-transmitting mesh bag (0.3 m × 0.4 m large organza bags; mesh size 0.5 mm, TtS Ltd, UK) secured around the pot using an elastic band and supported by its own seams so that it did not touch the plant leaves. For technique (2), a clip cage made from foam with a mesh opening (inside diameter 2.54 cm and thickness 9.5 mm, BioQuip

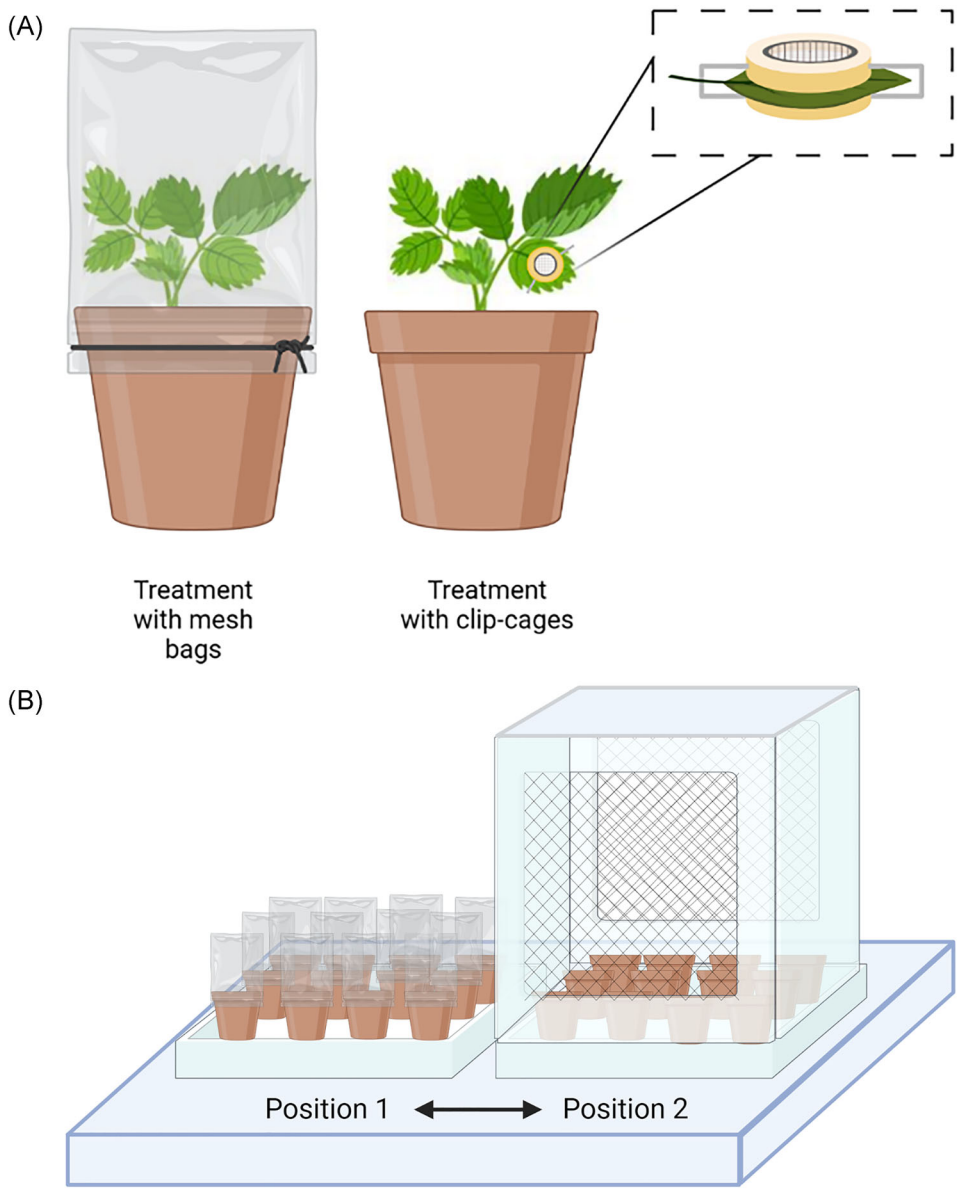

Fig. 1 (A) Illustration of the treatments used for the fitness assay. For the treatment with mesh bags, the aphid was transferred to the abaxial surface of one of the unfolded leaves, then the plant was covered by a mesh bag and secured by a rubber band. For the treatment with clip cages, the aphid was transferred to the abaxial surface of one of the unfolded leaves and secured onto it with two foam discs (with the side containing the aphid on the leaf being covered with microperforated polypropylene) on each side of the leaf using large clips. (B) Illustration of the experimental set-up. Created with BioRender.com.

products Inc., USA) was used to contain an aphid. The aphids were checked daily; in order to clearly see the aphid, the meshed bags were removed and replaced, and clip cages were opened and closed every day. The experiment comprised a randomized split-block design with five temporal blocks. Each block contained two trays of plants, to which containment treatment and position within the environmental chamber were assigned as random (Fig. 1B). Within each tray, 3 replicates of each clonal line (4 in total) were assigned at random to a plant position, giving a total of 12 plants per tray and 24 plants per temporary block. Therefore, both containment treatments were replicated 15 times for each aphid clone in total. Strawberry plants to which different aphid

Table 2 Description of aphid fitness parameters measured in the experiment.

Fitness parameter	Measurement or equation	Reference
Intrinsic rate of natural increase (r_m)	$\frac{0.738 \ln(Fec)}{d}$	Hu *et al.* (2018)
Mean relative growth rate (MRGR)	$\frac{\ln(W2)-\ln(W1)}{(t2-t1)}$	Castel & Berger (1993)
Time to reach reproductive adulthood (TTRA)	Number of days from birth to onset of reproduction	Hu *et al.* (2018)
Population doubling time (DT)	$\frac{\ln(2)}{r_m}$	Hu *et al.* (2018)

d = developmental time (time from birth to adulthood + 0.5), Fec = fecundity (number of nymphs produced in over the first d days of adulthood), $W1$ = Initial mean weight of nymphs on day 1, $W2$ = weight of a single nymph on day 5, ($t2-t1$) = days between the initial and final weighing.

containment methods had been applied were placed on plastic trays (57.2 cm × 38.8 cm × 5 cm, G16B, Garland Products Limited, England) and maintained under the same environmental conditions as previously described throughout the experiment.

The fitness bioassay was initiated by transferring three age-synchronized adult apterous aphids, 1–2 d post maturation, onto the abaxial surface of a young fully unfolded trifoliate strawberry leaf (clip cage treatment) or the crown of a strawberry plant (mesh bag treatment). For the clip cage treatment all the plants were maintained in an insect proof mesh cage (47.5 cm × 47.5 cm × 47.5 cm, BugDorm-4S4545) to prevent cross-contamination if insects escaped from their clip cage. For the mesh bag treatment, plants were not held within an insect proof mesh cage as each plant was already covered by the bags. After 24 h, all aphids except one were removed using a 000 paintbrush, leaving a single first instar nymph per clip cage or entire plant without being disturbed. Removed nymphs were weighed using a microbalance (XPR10 Ultramicrobalance, Mettler Toledo, Greifensee, Switzerland) and the mean initial first instar weight calculated. The remaining nymph was left to develop and weighed on day 5 to calculate the mean relative growth rate (MRGR). Clip cages and plants in mesh bags were monitored daily to record intrinsic rate of natural increase (r_m), time until reproductive adulthood (TTRA), and population doubling time (DT) (Table 2). The unit of replication was a single nymph per plant, whether enclosed using a clip cage or mesh bag. For each replicate block and treatment, clonal lines were ranked from 4 to 1 for each biological parameter, with 4 for the clonal line with highest fitness and 1 for the clonal line with the lowest fitness. Points for each biological parameter were summed to rank the replicates of each clonal line for their overall fitness, with higher numbers correlating with better fitness.

Statistical analysis

Cohens d (Cohen's d = [Mean_2 – Mean_1]/standard $\text{deviation}_{\text{pooled}}$) was calculated for the response variables in the bioassay to determine whether tray position (1 or 2) had an impact on aphid fitness. A value of <0.18 indicated that this was not the case and replicates from different trays could be pooled for subsequent analysis. This was further supported using interaction plots to visualize the effect of tray position on results. Due to the homogeneity of environmental conditions in the controlled environment room and, therefore, between and within blocks and plots, a complete randomized design analysis was used for the experiment. Statistical analyses were carried out using R version 4.2.0 (R Core Team, 2021). Prior to any analysis, data were tested to determine if they conformed to the key assumptions for parametric statistical tests. Data distributions were checked using the Shapiro–Wilk test while homogeneity of variance was assessed by the Bartlett test. All data met the parametric assumptions, and therefore two-way analyses of variance (ANOVA) was performed for each measurement considering experimental treatment (clip cages vs. mesh bag) and clonal line. Afterward, if ANOVA results indicated a significant effect of a factor or an interaction, pairwise comparisons using a Tukey's HSD *post hoc* test were used to separate the means and establish which groups were different. In the case of count data (TTRA), a multivariate general linear model (GLM) using a quasi-Poisson distribution was used to investigate the effect of containment treatment and clonal line. This was followed by a Sidak multiple pairwise comparison *post hoc* test to identify differences between means. Finally, for the overall fitness results, a multivariate general linear model (GLM) using a quasi-Poisson distribution was used to assess the effect of each containment method on aphid genotype fitness.

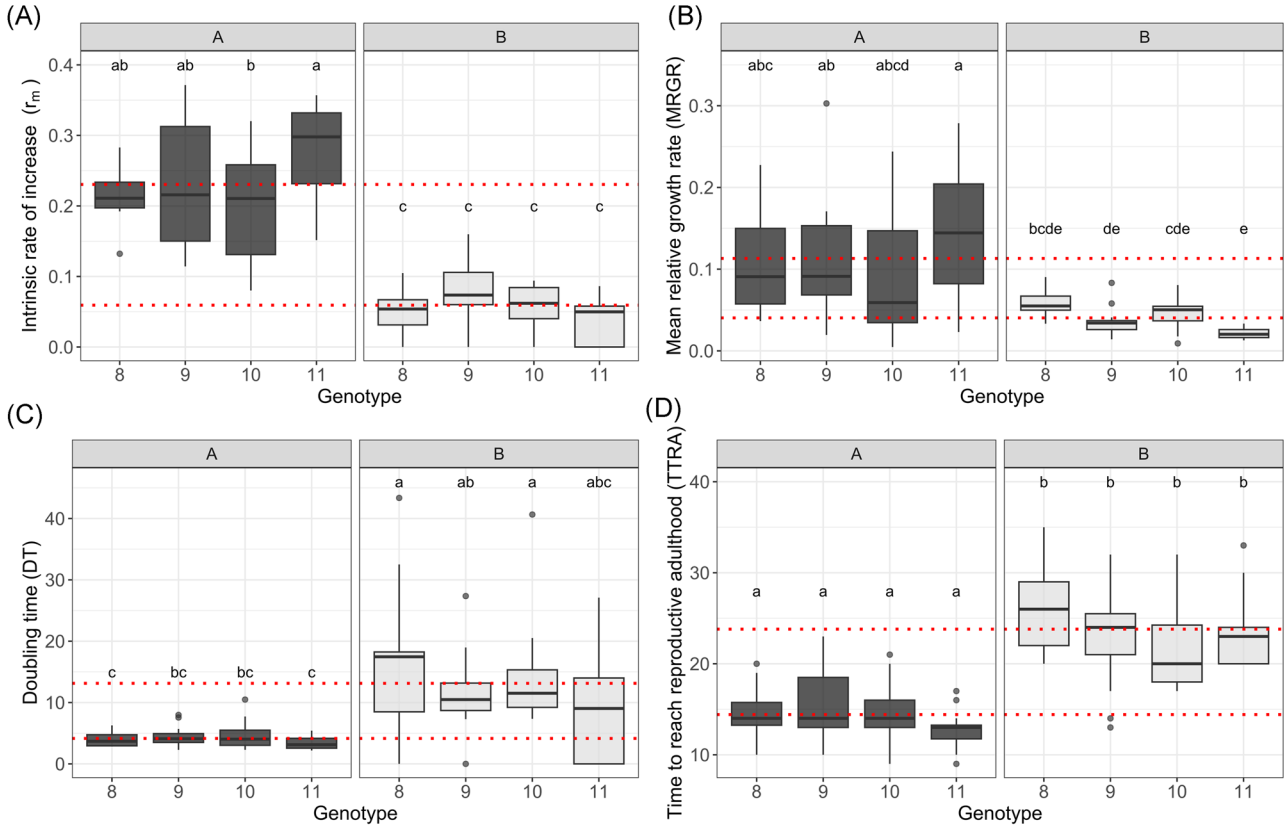

Fig. 2 Macrosiphum euphorbiae fitness parameters by genotype on different treatments (A) aphids enclosed on entire plants covered by mesh bags, and (B) aphids enclosed on leaves using clip cages. Red lines represent the overall mean for each treatment.

Results

Potato aphid genotypes and secondary endosymbionts

The four clonal lines represented four different *M. euphorbiae* genotypes (Table S3), which were distinct from the genotypes characterized previously (Clarke *et al.*, 2017) and therefore named as genotype 8 (HAU_1), genotype 9 (HAU_2), genotype 10 (HAU_3), and genotype 11 (HAU_4) following the numeration initiated by the James Hutton Institute (Gaynor Malloch *pers. comm.*). Two of all facultative endosymbionts that were tested for were identified in three of the potato aphid clonal lines. *Hamiltonella defensa* was identified in two lines (genotypes 9 and 11), while *Regiella insecticola* was identified in only one line (genotype 10).

Impact of clip cages and genotype on potato aphid fitness

Intrinsic rate of increase Intrinsic rate of increase (r_m) for all genotypes was more than two fold higher using mesh bagged whole plants than for individuals contained on leaves within clip cages ($F = 236.75$, df $= 1$, $P < 0.001$), with a mean value of 0.23 for the mesh bag treatment and 0.05 for the clip cage treatment. Aphid genotype also significantly impacted intrinsic rate of increase ($F = 2.90$, df $= 3$, $P < 0.05$), with genotype 11 showing a greater value than the other genotypes on whole plants (mean value of 0.29) and genotype 9 showing a greater value than the other genotypes in clip cages (mean value of 0.07). This resulted in a significant genotype-by-treatment interaction ($F = 3.28$, df $= 3$, $P < 0.05$) (Fig. 2A).

Mean relative growth rate Mean relative growth rate of the clones using mesh bagged whole plants was

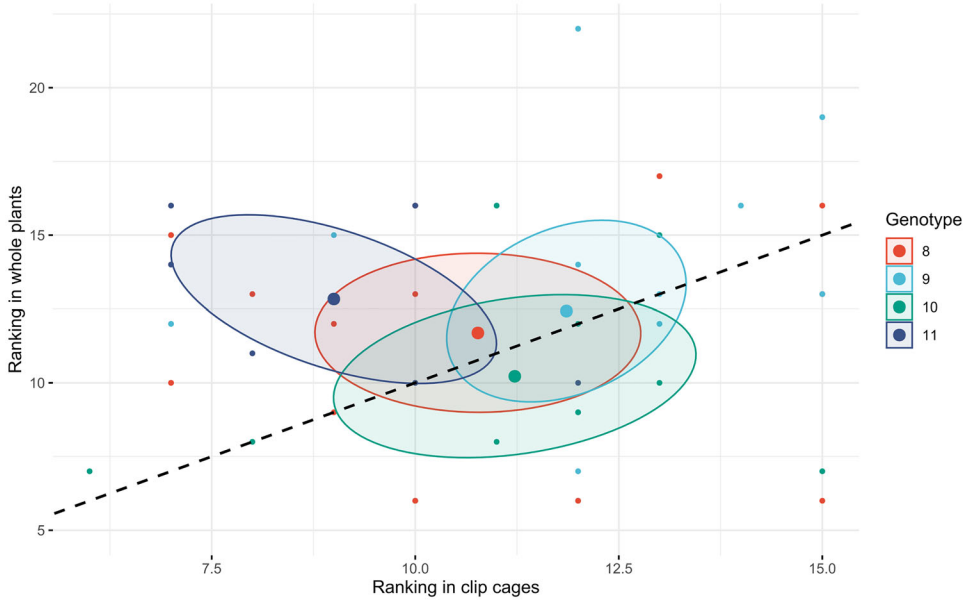

Fig. 3 The overall fitness ranking of four *M. euphorbiae* genotypes when contained on whole plants versus in clip cages. The means for each genotype is represented by a large dot of the same color. The black dashed line is a 1 : 1 line of equivalence.

almost three times higher than for individuals contained on leaves with clip cages ($F = 55.91$, df $= 1$, $P < 0.001$), with a mean of approximately 0.11 for the former and 0.04 for the latter. This fitness metric did not vary significantly between genotypes ($F = 0.49$, df $= 3$, $P > 0.05$). However, the genotype-by-treatment interaction was again significant ($F = 3.50$, df $= 3$, $P < 0.01$) (Fig. 2B).

Population doubling time Population doubling time was three times longer using clip cages on leaves than when using mesh bagged whole plants ($F = 47.40$, df $= 1$, $P < 0.001$), with a mean of 13 d for the clip cage treatment and of 4 d for the mesh bag treatment. In addition, population doubling time varied between genotypes ($F = 2.84$, df $= 3$, $P < 0.05$), with genotypes 9 and 10 showing a significantly greater value than the other genotypes on whole plants (mean value of 4 d) and genotype 8 showing a significantly greater value than the other genotypes in clip cages (mean value of 18 d). Nevertheless, here the genotype-by-treatment interaction was not significant ($F = 0.95$, df $= 3$, $P > 0.05$) (Fig. 2C).

Time to reach reproductive adulthood Time to reach reproductive adulthood doubled when using clip cages compared to bagged whole plants ($F = 131.05$, df $= 1$, $P < 0.001$), with individuals contained on leaves using clip cages taking 24 d compared to 14 d for those contained on whole plants using mesh bags. Genotype had a significant effect on time to reach reproductive adulthood ($F = 2.72$, df $= 3$, $P < 0.05$); however, no significant pairwise differences between genotypes were found when running the *post hoc* analysis. Finally, the genotype-by-treatment interaction was not significant ($F = 2.05$, df $= 3$, $P > 0.05$) (Fig. 2D).

Overall fitness Considering the overall fitness of the different potato aphid genotypes, no statistical differences between genotypes were found for the treatment with whole plants ($F = 0.30$, df $= 3$, $P > 0.05$). However, genotype 11 with a mean of 13.83 points had the highest fitness score when using whole plants covered with a mesh bag followed by genotype 10. On the other hand, genotype fitness varied significantly when using clip cages ($F = 2.86$, df $= 3$, $P < 0.05$), with genotypes 9 and 10 performing better with a mean of 11.86 and 11.63 points, respectively (Fig. 3).

Discussion

Containment method was found to influence the fitness of potato aphids. Using clip cages resulted in a significant reduction in aphid fitness relative to using bagged whole plants. This effect was observed for all the fitness parameters measured, indicating that containing aphids in clip cages impacts both their development and fecundity. Perhaps more interestingly, our results highlighted genotypic/clonal differences in the effects of containment method on potato aphid fitness. To the best of our knowledge this study provides the first report of the influence

that clip cage containment has on aphid fitness while also providing insight on how genotypic/clonal variation impacts aphid fitness parameters.

Entomologists have long used clip cages to contain aphids to measure fitness parameters as a proxy for aphid fitness (Dixon & Wratten, 1971). This technique is widely recognized as one of the most useful and space-efficient methods for studying aphid traits (Haas *et al.*, 2018). However, in this study, we show that clip cages can affect aphid fitness in laboratory bioassays by reducing the level of widely used fitness metrics such as intrinsic rate of increase and mean relative growth rate as well as increasing the time to reach reproductive adulthood and estimated population doubling time. This suggests that clip cages can generate misleading results for aphids, when used in fecundity studies, plant resistance research, or natural history descriptions. In addition, other invertebrates confined in clip cages may also be negatively impacted. Therefore, the use of clip cages, or similar techniques, can have significant repercussions in applied entomology. For example, pest management decisions in which poor aphid fitness may be mistakenly attributed to varietal plant resistance, leading to poor plant breeding recommendations and insecticide use decisions. However, similar studies with other aphid species have shown different results, suggesting that the effect of clip cages might be species dependent. For example, using English grain aphid (*S. avenae*), a study showed that clip cages did not have an adverse effect on reproduction and development (Kou *et al.*, 2022). Similarly, for peach potato aphid (*Myzus persicae* Sulzer) and corn leaf aphid, no significant differences in fecundity were observed when a comparison of using clip cages or whole plant cages was completed (Mowry, 1993).

The impact of containment on aphid fitness could be caused by their differential effects on the physiological and physical characteristics of the plant leaf or whole plant (Stamp & Bowers, 1994; Moore *et al.*, 2003), which in turn can affect nutrient availability in the tissues and therefore have a differential influence on aphid fitness itself. In addition, it has been noted that opening and closing clip cages (which happened daily for this experiment) can disturb aphid feeding activity or induce defense pheromone production, which can both generate additional metabolic costs for the aphids (i.e., relocation of resources at the expense of growth and reproduction), negatively impacting aphid fitness (Haas *et al.*, 2018). Individual aphid species may have specific feeding site preferences (Nalam *et al.*, 2021). Bird cherry-oat aphid and mealy cabbage aphid (*Brevicoryne brassicae* L.), for example, prefer feeding sites close to the soil surface or upper leaves depending on the season and plant developmental stage (van Emden & Harrington, 2017). Also, peach potato aphid shows a strong preference for young leaves with higher nutritional quality compared to older leaves that typically have higher concentrations of toxic metabolites (Cao *et al.*, 2018). To our knowledge, there is no information available for potato aphid feeding site preference on plant hosts other than lettuce, on which they prefer to feed on outer leaves (Shrestha *et al.*, 2017). Nevertheless, the potato aphids used in our study preferred to feed on unexpanded leaves, young stems, or runners when provided with a choice in bagged whole plants. This observation is supported by observations by agronomists who have previously described potato aphids as being typically found on unexpanded leaves or flower/fruit stalks on different plant hosts (Taylor, 2013). It may then be expected that aphid species that prefer to feed on the same part of the plant where a clip cage is typically placed (i.e., fully expanded leaves) would exhibit optimal fitness compared with species usually found on other plant parts or different leaf developmental stages. Therefore, this information should be considered when selecting a technique and plant part for containing different species of aphids to perform fitness assays.

Aphid clonal/genotypic differences in fitness were observed under each containment treatment. Although aphid facultative symbionts have been reported to affect aphid fitness (Kaech *et al.*, 2022; Liu *et al.*, 2023), there was insufficient replication of clonal lines with different symbiont infections to detect any effects on potato aphid fitness in this study. However, genotypic/clonal variability in aphid fitness was particularly pronounced when using clip cages. This might be explained by clonal/genotypic differences in the aphid response to plant/leaf stress status (Srisakrapikoop *et al.*, 2021) or the ability to exploit a specific feeding site. Although there is no information available on either genotypic or endosymbiont influence on feeding site preference for any aphids, it could be hypothesized that if feeding sites differ physically and chemically and the insects are restricted to a specific part of the plant (i.e., the leaf in the case of clip cages), intraspecific variation in aphid preference could greatly impact aphid fecundity and body mass as observed in this study. Another possible explanation is that potato aphid genotypes may differ in their propensity to react to external stimuli, as has been shown for the presence of natural enemies in pea aphids (*A. pisum*) (Braendle & Weisser, 2001; Muratori *et al.*, 2014). For potato aphids, typical defense behaviors center around cessation of feeding followed by walking away (Humphreys *et al.*, 2021a), but to our knowledge, no information on clonal variation in defensive behaviors has been reported to date. As opening and closing clip cages can result in

excessive leaf movement and vibrations detectable by an aphid as sensory information related to the presence of predators or parasitoids (Nelson, 2007), the use of clip cages could cause feeding cessation and could also trigger energetically costly defensive behaviors like dropping or producing alarm pheromones (Harrison & Preisser, 2016). Vibrations have been linked to a higher probability of an aphid withdrawing its stylet and dropping from the plant (Gish, 2021), which can have an impact on aphid fitness as finding and accepting another feeding site is energetically costly (Nelson, 2007). This can be exacerbated if the disturbance is repeated almost daily like in the case of experiments using clip cages. However, it is important to note that a limitation of this study is that due to the experimental design, it is not possible to tease apart the physical disturbance from checking the clip cages and possible damage to plant tissue from the spatiotemporal confinement of the aphid to a single part of the host plant. This disturbance might interact with genotypic variation in physiological or biochemical traits. Aphid salivary effector proteins, for example, have been described to vary in their fitness effect on aphids by modulating reproduction (Elzinga *et al.*, 2014); little is known, however, about intraspecific variation in these proteins in the potato aphid and any effects on the aphid–plant interaction (Jonckheere *et al.*, 2016). Other genes though to relate to aphid sensory functions and detoxification pathways have been shown to vary in representation between potato aphid genotypes, but their functional role remains to be confirmed (Whitehead, 2019). On the other hand, aphids maintain relationships with secondary endosymbionts that can confer fitness benefits or costs to themselves. For example, the presence of *H. defensa* has been proven to increase the fitness of the Indian grain aphid (*Sitobion miscanthi* Takahashi), but the presence of both *H. defensa* and *R. insecticola* leads to significant fitness costs in corn leaf aphids (Liu *et al.*, 2023). Therefore, clonal fitness variation in response to confinement method may have significant repercussions in studies using clip cages, especially if the populations are not characterized (genotyped and screened for endosymbionts) or conclusions are drawn from a single aphid clonal background.

Our study indicates that potato aphids have greater fitness when reared on whole plants compared with clip cages, which are a standard method for insect laboratory bioassays. We also identified clonal variation in the way the clip cage confinement affects the fitness parameters, which we hypothesize could be linked to aphid genetic variation in important aphid physiological and behavioral aspects, such as feeding site preference and molecular processes at the aphid–plant interface, and the interactive effects of clip cages effects on plant physiology. These results should be considered when deciding whether to use clip cages for aphid fitness bioassays. They also open the door for subsequent studies on genotypic/clonal responses to different experimental techniques and on genotypic/clonal influence of feeding site preference. Special care should be taken when interpreting and comparing results from clip cage studies with those obtained using other techniques and using the information for pest management decisions. It should also be stressed that clip cages must be at least complemented with whole plant data to have a more rounded understanding of any traits measured.

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Disclosure

The authors declare that there are no relevant interests to disclose.

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Supporting Information

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

Table S1. Microsatellite primer sequences, fluorochromes labels in parentheses. Source (Raboudi *et al.*, 2005).

Table S2. Diagnostic PCR primer sequences used for detecting facultative endosymbiont infection.

Table S3. Allele sizes from seven microsatellite loci amplified from the four *M. euphorbiae* clonal lines sampled from strawberry crops.