Analysis of the impact of extrinsic and intrinsic factors on aphid locomotion using a novel machine-learning based system to track insect behavior

El-Sayed Baz, Cissy Huygens, Federica Calevro, Patrick Callaerts

To cite this version:

El-Sayed Baz, Cissy Huygens, Federica Calevro, Patrick Callaerts. Analysis of the impact of extrinsic and intrinsic factors on aphid locomotion using a novel machine-learning based system to track insect behavior. Entomologia Generalis, 2025, 45 (2), pp.381-389. ⟨10.1127/entomologia/2025/2876⟩. ⟨hal-05128815⟩

Entomologia Generalis
Published online March 2025

Analysis of the impact of extrinsic and intrinsic factors on aphid locomotion using a novel machine-learning based system to track insect behavior

El-Sayed Baz[1,3,*], Cissy Huygens[1], Federica Calevro[2], Patrick Callaerts[1,*]

1 Laboratory of Behavioral and Developmental Genetics, Department of Human Genetics, KU Leuven, University of Leuven, B-3000, Leuven, Belgium
2 INRAE, INSA Lyon, BF2I, UMR 203, 69621, Villeurbanne, France
3 Zoology Department, Faculty of Science, Suez Canal University, 41522 Ismailia, Egypt
* Corresponding authors: patrick.callaerts@kuleuven.be; elsayed.baz@kuleuven.be

With 4 figures

Abstract: Locomotion is central to a broad range of animal behaviors, and its quantification and analysis can provide insight into the factors that influence and regulate those behaviors. In the current study, we used a novel custom-made 3D–printed circular arena alongside open-source idtracker.ai software to investigate how the pea aphid *Acyrthosiphon pisum* adapts locomotor behavior in response to extrinsic (arena size and group size) and intrinsic factors (starvation – hunger). The results revealed that the locomotor activity of solitary aphids is higher in larger arenas. In addition, solitary aphids walked longer distances at high speeds than aphids in groups. Starved aphids displayed starvation-induced hyperactivity that remained at high levels over time, whereas fed aphids displayed slower, progressively increasing movement. Furthermore, unlike in fed aphids, tonic immobility in starved aphids was notably reduced or absent. This study advances our understanding of aphid behavioral plasticity and reveals the potential of machine learning-based tracking as a scalable approach to characterize insect behavioral adaptations and survival strategies, which could lead to novel applications in pest management.

Keywords: *Acyrthosiphon pisum*; behavioral arena; locomotor activity; starvation; behavioral pattern; plasticity

1 Introduction

Locomotion is central to a broad range of animal behaviors. Consequently, its quantification and analysis can provide insight into the extrinsic and intrinsic factors that influence and regulate behaviors such as dispersal, food seeking, predator escape, reproduction, but also spread of disease in cases of disease vectors. An important group of insects are aphids, pest insects with a major impact on agricultural crops (Chen 2024). Aphids cause direct damage through sap feeding. Indirect damage stems from aphids acting as vectors of plant viruses and from increased molding following honeydew deposition by the aphid (van Emden & Harrington 2007). Dispersal of aphids is an important variable determining the impact of the aphids on any given crop since a single aphid can quickly establish a new colony through parthenogenetic reproduction (Zhang et al. 2016). Consequently, insight in dispersal dynamics is important for optimizing control methods. Aphid dispersal can either be long distance or short distance by winged (alates) and wingless individuals, respectively. The study of aphid dispersal has primarily focused on alates. Alates are produced in response to overcrowding and declining nutritional value of the host plant (Müller et al. 2001) and are able to cover long distances to find new host plants (van Emden & Harrington 2007). By contrast, the impact of wingless aphids on dispersal is less well studied. Short distance movement has been implicated in virus transmission within fields (Hodgson 1991; Irwin et al. 2007). It has been shown that different aphid species are able to cross a soil surface to the nearest plant in a relatively short time (Alyokhin & Sewell 2003; Ben-Ari et al. 2015; Diaz et al. 2012). During the transfer, aphids encounter various challenges, including predators, field size, population size, and periods of starvation (Irwin et al. 2007; Roitberg et al. 1979). However, little is known about the factors (extrinsic and intrinsic) that

DOI: 10.1127/entomologia/2025/2876

E. Schweizerbart'sche Verlagsbuchhandlung, 70176 Stuttgart, Germany, www.schweizerbart.de

affect aphid locomotion even though it can be expected that this knowledge will inspire control strategies.

In the current study, we focused on the pea aphid *Acyrthosiphon pisum*. Not only is the pea aphid an important pest of legume crops and an important vector of plant viruses, it has also emerged in recent years as a model aphid thanks to the high quality of the genome assembly and annotation and the establishment of methods that make it experimentally tractable (Calevro et al. 2019; IAGC 2010). Previous studies showed how *A. pisum* adapts to different environmental stressors (Brodsky & Barlow 1986; Ribeiro Lopes et al. 2022; Roitberg & Myers 1979), and how it interacts with its host plants (Sochard et al. 2019). Aphid locomotion, on the other hand, is much less studied, even though it is a core behavior linked to dispersal and thus the potential for causing damage (Zhang et al. 2016).

Recent advances in machine learning-based tracking and 3D printing have significantly enhanced tools for analyzing animal locomotion in ways that were not previously technically possible (Luxem et al. 2023). Given their economic importance, characterizing aphid locomotion and identifying factors that regulate this can open avenues for improved aphid control. Thus, in this study, we first established an integrated method using customized 3D–printed behavioral arenas and open-source idtracker.ai software (Romero-Ferrero et al. 2019) to track the movement of aphids. We next investigated how aphids adapt their locomotor activity in response to extrinsic (arena size and group size) and intrinsic (hunger upon starvation) factors. We found that single aphids increased their locomotor activity in larger arenas compared to smaller ones. Moreover, single aphids moved more than those in groups. Additionally, long-term starvation resulted in sustained hyperactivity and absence of tonic immobility. Our findings highlight the complex interplay of these factors and their role in aphid survival and behavioral adaptations. Furthermore, the combination of uniform arenas and machine learning to analyze locomotion represents a scalable approach that can be used for (semi) high-throughput studies of aphid locomotion to improve control strategies.

2 Materials and methods

2.1 Insects

All aphids (*A. pisum* (Harris)) were from the LL01 line, characterized by its sole symbiosis with the primary endosymbiont *Buchnera aphidicola*. Aphids were reared on young broad bean plants (*Vicia faba* L. cv. Aguadulce) in aerated transparent plastic cages (45 × 40 × 33 cm) in an environmental incubator (Panasonic MLR 352H-PE, Gunma, Japan) at 20 °C, 60% RH and a photoperiod alternating between 16 hours light and 8 hours darkness with zeitgeber time zero (ZT0; the time the light went on) at 7:00 am. This ensured the maintenance of strictly parthenogenetic matrilines with genetically identical aphids to reduce behavioral differences between individuals.

Wingless adult pea aphids were used for all experiments. Synchronized wingless aphids were produced as described by Sapountzis et al. (2014). About 100 winged adults were randomly selected from the stock cultures using a small paintbrush. These aphids were then carefully placed on seedlings, enabling them to produce nymphs before being removed after 24 hours. The resulting N1 (1st-instar) nymphs were allowed to grow for nine days to reach adulthood (stage A9).

2.2 Behavioral arena and setup

The locomotor behavior of aphids was evaluated in a custom-made circular tracking arena designed using the onshape web-based CAD software (http://www.onshape.com) and 3D printed in-house (see Fig. 1A for schematic representation). We were motivated to produce this type of arena by the problems encountered with initial experiments where we used Petri dishes: loss of aphids due to the absence of an integrated water barrier, difficulties imaging aphids when moving vertically on the Petri dish wall, inadvertent movement of Petri dishes when manipulating the setup. The creation of a custom-made arena addressed all these problems while the additional introduction of a barrier also eliminated a possible effect on aphid locomotion of visual cues stemming from conspecifics. Furthermore, our design is readily scalable for (semi) high-throughput analyses. The arenas were positioned on the middle shelf of a climate-controlled chamber (20 °C, 60% RH, Panasonic MLR 352H-PE). A customized broad-spectrum lighting system was placed on the bottom shelf of the recording chamber below the tracking arenas. It consisted of a total length of 120 cm white LED strip (White dimmable, waterproof LED strip; SMD5050-300, LEDLightsWorld). The LEDs were mounted on the outer walls of a square aluminum tray (30 × 30 × 5 cm) to directly illuminate the walls of the recording chamber and thus indirect, reflected diffused light of approximately 400 lx atop the behavioral arenas. A high-definition camera (e.g., Sony HDR-CX240E or SM-A202F camera) was positioned above the tracking arenas. The CAD files for the 3D–printed parts are available from the accompanying repository at https://github.com/Sayedbaz/Aphid_Tracking.

2.3 Experimental design and procedures

The impact of extrinsic (arena size, group size), and intrinsic (hunger – starvation) factors on aphid locomotion was determined. For arena size experiments, single aphids were tested at ZT2 (i.e., two hours after lights-on) in three different arena sizes: 30, 55, and 70 mm in diameter (Fig. 1B). For group size experiments, at ZT2, aphids were tested in the same arena size of 55 mm diameter with groups of three sizes: 1, 5, or 10 individuals per arena. For starvation experiments, at ZT2, aphids were randomly partitioned into two equal sized batches. The first batch of aphids corresponded

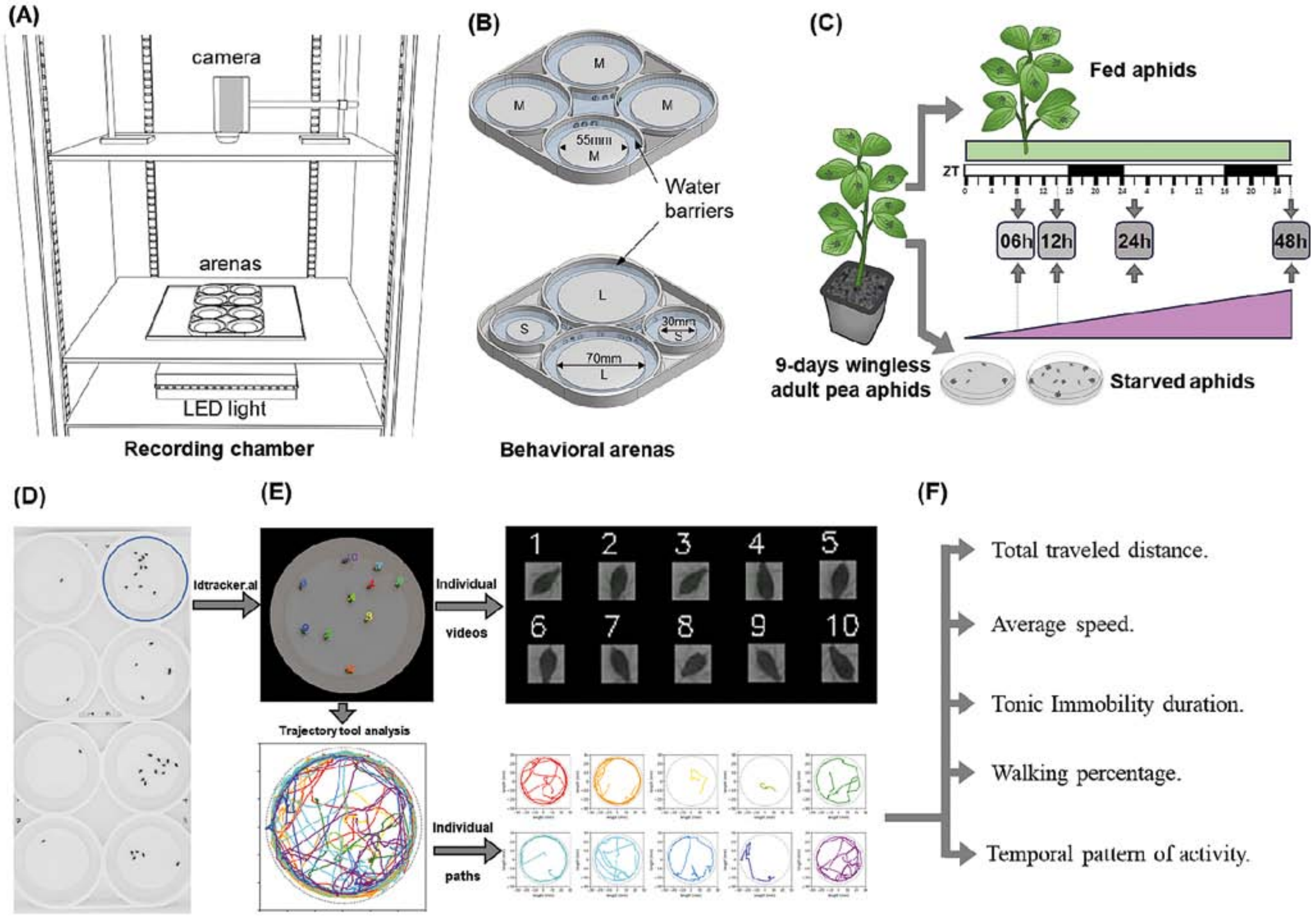

Fig. 1. Schematic representation of the experimental design, behavioral assay, and tracking methods.
(A) Diagram of the different components of the experimental setup. The behavioral arenas for tracking aphid locomotion were placed in the middle of a climate-controlled recording chamber. The arenas were illuminated from below with indirect white light and imaged from above with a digital video camera. **(B)** Examples of custom-made behavioral arenas of different sizes for recording aphid locomotor activity. Water barriers surrounded each arena to prevent aphids from escaping and were enclosed by high profiles to block visual contact between different arenas. **(C)** Starvation protocol. For groups that underwent the starvation treatment, aphids were transferred to Petri dishes containing a wet filter paper in groups of 5 or 10 individuals. For groups without starvation, the aphids were transferred to new plants. Aphids were starved for 6, 12, 24, or 48 hours before the recording their locomotor activity behavior. Aphids were maintained under LD 16:08 photoperiod conditions, and locomotor activity was monitored during the light phase, indicated by the white bars, while the black bars indicate the dark phase. **(D)** A snapshot from a video of 8 behavioral arenas. **(E)** An example of tracked video and trajectory analysis. Top: a snapshot of a single arena containing 10 aphids and the collage of individuals tracked with idtracker.ai software. Bottom: representative trajectory plots for 10 aphid movement during 30 minutes and the independent trajectories for each individual within the behavioral arena. **(F)** Trajectory data were analyzed to quantify the distance traveled, average speed and tonic immobility durations, and walking percentage in 30-minute video recordings. Additionally, the temporal dynamics of the movement in 6 successive 5-minute intervals over 30 minutes were determined.

to starved groups, while the second batch of aphids represented fed control groups. Starved aphids were distributed over about 20 Petri dishes (60 mm diameter, 15 mm height; 628102, Greiner Bio-One, Frickenhausen, Germany), without food, in replicates of 5 or 10 individuals per dish. The lid of the Petri dishes was fitted with a single moist filter paper of 55 mm (Whatman, 1001-055, Fisher Scientific, Brussel, Belgium) to prevent desiccation. Upon addition of the aphids, the Petri dishes were inverted so that the lid with the filter paper formed the bottom. The aphids were subsequently starved for 6, 12, 24, or 48 hours prior to recording. The control groups of fed aphids were returned to new fresh broad bean plants to assess baseline locomotor behavior at different time points (Fig. 1C). Starved and fed aphids were maintained in the same climate-controlled chamber until testing. At each time point, starved and fed aphids were tracked simultaneously in arenas of 55 mm in diameter and with differently sized groups: 1, 5, or 10 individuals.

For all behavioral experiments, aphids were transferred carefully to the behavioral arenas. After transferring the aphids into the recording arena, they first exhibited tonic immobility (TI) before initiating walking as shown before

(Humphreys & Ruxton 2018). Following an acclimation period of 5 minutes, locomotor activity was recorded for 30 minutes at 30 frames per second (fps). Each treatment was assessed using four independent experiments with different synchronized batches of aphids. The order of treatments was distributed randomly across the arenas, ensuring that each condition was never exclusively tested in the same arena in different sessions. After each experiment, the behavioral arenas were thoroughly cleaned with ethanol, rinsed overnight with water, and allowed to dry prior to the next experiment.

2.4 Video processing and trajectory analysis

To determine aphid trajectories, the recordings were processed offline using the free open-source idtracker.ai software (Version 5.2.7) (Romero-Ferrero et al. 2019) that ran with NVIDIA RTX A500 Laptop GPU. To prevent identity transfer between individuals of multiple arenas, each tracked session included data of a single region of interest (ROI) that represents trajectories of individuals in the same behavioral arena (Fig. 1D). The validated tool of idtracker.ai was used to ensure that the identity of every single aphid was maintained throughout the experimental trial. Additional analysis was performed with the Python library trajectorytools (Heras et al. 2019) to extract position coordinates for each individual in the arena (Fig. 1E). While loading trajectories, we used sigma value = 1 to smooth the trajectory data. The distance was determined using the formula: $\mathrm{SQRT}[(x2–x1)^2 + (y2–y1)^2]$, where (x1, y1) and (x2, y2) are the position coordinates between two points. The tracking data were converted from pixel coordinates to real distances in mm. While tracking at 30 fps was sufficient to preserve the identity of each aphid with idtracker.ai software, recordings at higher fps led to noise that could artificially create incorrect distances (Nilsen et al. 2013; Romero-Ferrero et al. 2019). Therefore, we determined a movement threshold of 0.15 mm (equivalent to 0.3 mm/s) between consecutive frames at a 2 fps range to identify significant step lengths and filter out noise (Fig. S1).

Duration of TI and percentage of walking behavior were recorded. TI duration corresponded to the time needed to initiate the first significant movement after starting the recording. The walking percentage corresponded to the proportion of time spent in motion relative to the total duration (i.e., 30 minutes). Finally, an interaction network was constructed to quantify pairwise interactions between starved or fed aphids using a method similar to the one described in Romero-Ferrero et al. (2019). The interaction matrix was then used to construct a directed graph representation of the interaction network using the NetworkX library.

2.5 Statistical analysis

Normal distribution of datasets was assessed using the Shapiro-Wilk test and D'Agostino–Pearson test. Comparisons between the two groups were made with Mann–Whitney U tests for non-parametric data. Comparisons between more than two groups were made with ANOVA Kruskal–Wallis tests combined with the Dunn *post-hoc* test to detect differences between experimental groups. Proportion data were transformed using arcsine square root for the analysis of the walking percentage. For repeated measures of interval analysis, the Friedman or One-way ANOVA RM tests, as appropriate, were used, followed by Dunn's or Dunnett's multiple comparisons tests, respectively.

3 Results

3.1 Influence of arena size on locomotor activity of solitary aphids

Since the arena can impact movement properties (Scharf et al. 2024), we first determined the effect of arena size on aphid locomotion. The results revealed that overall locomotor activity of solitary aphids was lowest in the smallest arena (Kruskal-Wallis: $H = 9.797$, $df = 2$, $P = 0.007$) (Fig. 2). Specifically, total distances traveled and average speeds in large arenas (⌀70 mm) were significantly higher compared to small arenas (⌀30 mm) (Dunn's post hoc: ⌀70 mm vs. ⌀30 mm $P = 0.005$) (Fig. 2B, 2C). No significant differences were found in the proportion of time aphids spent walking or of TI across all arena sizes (Kruskal-Wallis: $H = 0.390$, $df = 2$, $P = 0.823$ and $H = 1.836$, $df = 2$, $P = 0.399$, respectively) (Fig. 2D, 2E).

Interestingly, over six consecutive 5-minute intervals, the temporal dynamics of movement activity revealed distinct patterns for the three groups (Fig. 2F–I). Initially, all groups displayed similar levels of distances traveled. Over time, aphids in arenas of ⌀70 mm (Fig. 2F) showed prolonged significant increases in locomotor activity as they spent more time in the arena (Friedman: $\chi^2 = 37.764$, $df = 5$, $P < 0.001$). Aphids in arenas of ⌀55 mm (Fig. 2G) showed slower, progressively increasing movement over time (Friedman: $\chi^2 = 25.193$, $df = 5$, $P < 0.001$). In contrast, aphids in arenas of ⌀30 mm (Fig. 2H) showed comparable low level locomotor activity across all intervals (Friedman: $\chi^2 = 5.072$, $df = 5$, $P = 0.407$). Overall, the locomotor activity of single aphids was higher in large compared to small arenas (Fig. 2I).

3.2 Influence of group size on locomotor activity of aphids

Aphids typically live in colonies but will disperse under unfavorable conditions such as overcrowding or poor nutrition (van Emden & Harrington 2007; Ben-Ari et al. 2015). We therefore first characterized the impact of group size, an important extrinsic factor, on locomotion. Solitary aphids traveled significantly longer distances (Fig. 3B), moved at higher speeds (Fig. 3C), and spent more time walking (Fig. 3D) compared to the 5 and 10 individuals in an arena of the same size (⌀55 mm) (Kruskal-Wallis: distance

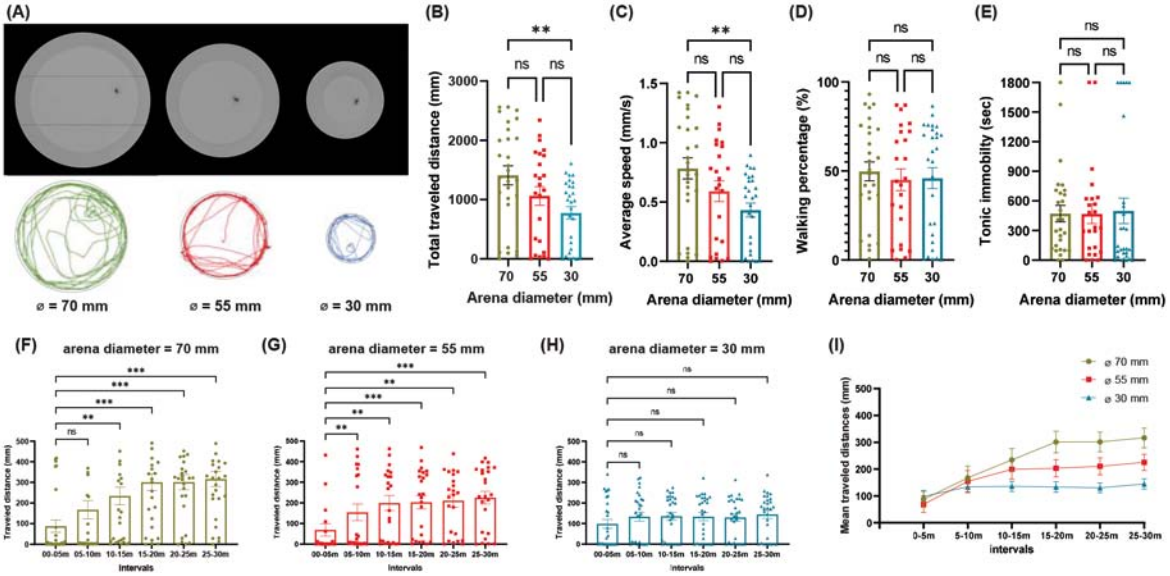

Fig. 2. Influence of arena size on locomotor activity of aphids.
(A) Top: snapshots of behavioral arenas of three diameters: 70 mm, 55 mm, and 30 mm, each containing a single aphid. Bottom: representative examples for trajectories of a solitary aphid per arena in three different-sized arenas for 30 minutes. The grey dotted circle is the boundary of the behavioral arenas. Differences in total distances traveled **(B)**, average speed **(C)**, walking percentage **(D)**, and tonic immobility duration **(E)** in three different arena sizes. **(F–H)** Comparisons of traveled distances of aphids tested in three arena sizes in 6 consecutive 5-minute intervals over 30 minutes. **(I)** Comparison of mean traveled distances for all aphids tested in different arena sizes. Plots represent mean ± SE, and each dot represents data measurement for one individual. (⌀70 mm, n = 28), (⌀55 mm, n = 24) and (⌀30 mm, n = 28). (B–E), Kruskal-Wallis tests followed by Dunn's multiple comparisons test. (F–H), RM Friedman tests followed by Dunn's multiple comparisons test. *Asterisks* represent statistical significance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, not significant. For statistical details, see Table S1.

H = 11.393, df = 2, $P = 0.003$, speed H = 11.393, df = 2, $P = 0.003$, and walking percentage H = 11.609, df = 2, $P = 0.003$). Groups of 5 and 10 individuals showed no significant differences in total traveled distances, speeds, and walking percentages. Furthermore, in all three groups, there were no significant differences in TI duration (Kruskal-Wallis: H = 3.627, df = 2, $P = 0.163$) (Fig. 3E).

Analysis of the temporal dynamics of locomotor activity showed that the distances traveled in the first interval of five minutes were the same in all three group sizes (Kruskal-Wallis: H = 3.423, df = 2, $P = 0.181$). Then, aphids showed increases in total locomotor activity (Fig. 3F–I). The longer distances traveled over time were more pronounced in the single aphid group (Friedman: $\chi^2 = 32.568$, df = 5, $P < 0.001$) (Fig. 3F). Groups of 5 aphids (Fig. 3G) displayed movement patterns similar to the groups of 10 aphids (Fig. 3H), with gradual increases over time but lower than the single aphids (Fig. 3I).

3.3 Influence of starvation on locomotor activity of aphids

We next determined the effect on locomotion of starvation, i.e. hunger, a highly relevant intrinsic factor. We found that starved aphids of different group sizes exhibited starvation-induced hyperactivity after prolonged periods of starvation. However, when comparing the locomotor activity of the three different-sized starved groups within each starvation duration, we found no significant differences, except between single-starved aphids and groups of 10 starved aphids after 48 hours (Fig. S2). Therefore, we selected a group of 10 aphids per arena further to investigate the effects of starvation on locomotor activity.

We observed that starved aphids consistently display high locomotion (total distance travelled, average speed, walking percentage) irrespective of the duration of starvation (6, 12, 24, 48 hours). We also found that with increasing starvation duration, the time of tonic immobility (TI) was highly significantly reduced after 6 hours of starvation (Mann-Whitney: U = 444, $P = 0.001$) (Fig. 4D), and essentially absent after 12 hours or more of starvation (Mann-Whitney: U = 255, $P < 0.001$) (Fig. 4D).

When comparing the locomotion of starved aphids with control fed aphids, we noticed that after 6 hours of starvation, no significant differences were observed for total distance traveled (Fig. 4A) and speed (Fig. 4B) (Mann-Whitney: U = 593, $P = 0.095$). However, aphids starved for 6 hours walked significantly more in 30 minutes than controls (Mann-Whitney: U = 451, $P = 0.002$) (Fig. 4C). At 12 hours of starvation, the differences between starved and fed aphids in total traveled distances and walking percentages were significant (Mann-Whitney: U = 555, $P = 0.04$ and U = 305, $P < 0.001$, respectively). After 24 h and 48 h of starvation,

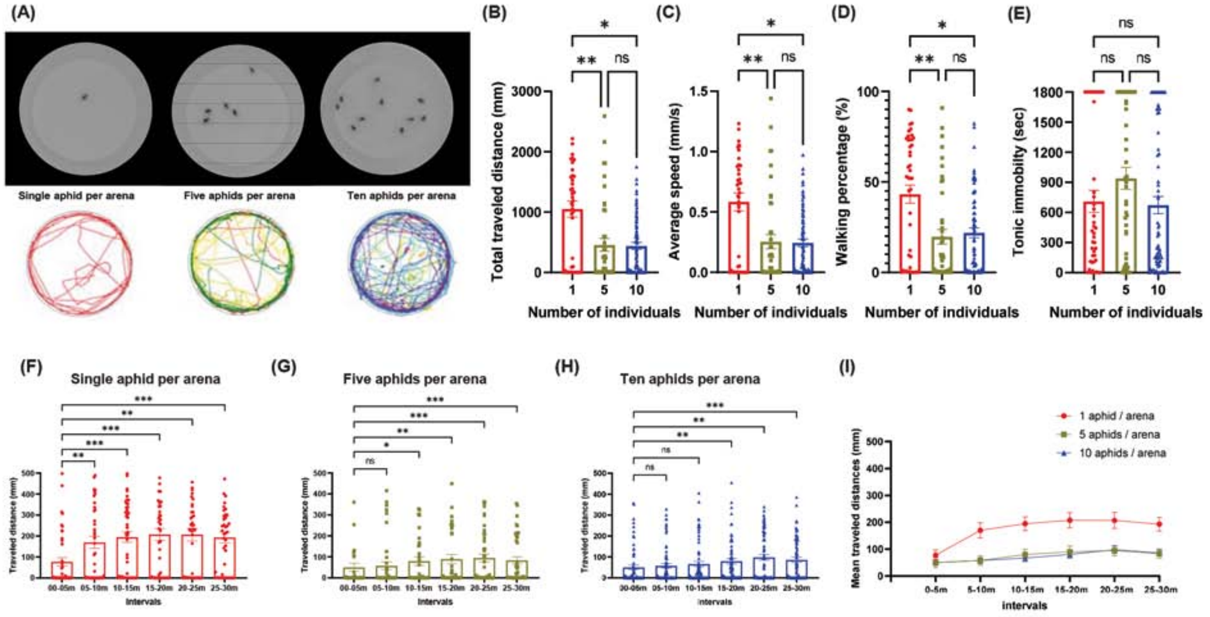

Fig. 3. Influence of group size on locomotor activity of aphids.
(A) Top: snapshots of behavioral arenas, with a diameter of 55 mm, illustrating the three group sizes (1, 5, and 10 aphids per arena). Bottom: representative examples for trajectories of 1, 5, and 10 aphids, corresponding to tested individuals in three groups during 30 minutes. The grey circle represents the boundaries of the behavioral arenas with a diameter of 55 mm. The grey dotted circle is the boundary of the behavioral arenas. Locomotor activity was recorded 2 hours after lights were turned on (ZT02). The movement was tracked using a digital camera, and data were analyzed for total distance traveled **(B)**, average speed **(C)**, the proportion of time spent walking **(D),** and tonic immobility duration **(E)** in groups of three different sizes. **(F–H)** Temporal progression of traveled distances of aphids tested in three arena sizes in 6 successive 5-minute intervals over 30 minutes. **(I)** Comparisons of mean traveled distances for all aphids tested in three group sizes. Plots represent mean ± SE, and each dot represents data measurement for one individual. (Single aphid per arena, n = 43), (Five aphids per arena, n = 43), and (Ten aphids per arena, n = 68). (B–E), Kruskal-Wallis tests followed by Dunn's multiple comparisons test. (F–H), RM Friedman tests followed by Dunn's multiple comparisons test. *Asterisks* represent statistical significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant. For statistical details, see Table S1.

the differences in all variables (overall locomotor activity, speeds, and walking percentages) (Fig. 4A, B, and C, respectively) of starved aphids compared to the control groups were highly significant (Mann-Whitney: $P < 0.001$). Part of the reason for the much larger differences between starved and control aphids after 24 and 48 hours of starvation is the fact that the fed aphids showed much more activity at 6 and 12 hours. This made us wonder whether there might be an effect of circadian rhythmicity. When comparing total distance travelled by fed aphids at ZT8 (corresponding to 6 hours starvation and control) and ZT14 (corresponding to 12 hours starvation and control) to ZT2 (corresponding to 24 and 48 hour starvation and control), we found that locomotion of fed aphids at ZT8 and ZT14 is higher (Fig. S3). We conclude that these differences in activity dampen to some extent the observed differences between fed and starved aphids at 6 and 12 hours. Nonetheless, the main observation remains that starvation induces hyperactivity in aphids after 6, 12, 24 and 48 hours of starvation.

We further analyzed the temporal dynamics of locomotor activity in fed and starved aphids during 30-minute recording sessions (Fig. S4). We found that aphids starved for prolonged periods exhibited elevated starvation-induced hyperactivity with no significant differences between consecutive intervals. In contrast, fed aphids showed gradual increases in movement over time. Furthermore, comparison of starved and fed aphids at each time point in the 6 hours starvation condition revealed no significant differences between starved and fed aphids throughout all intervals (Fig. S4B). After 12 hours of starvation, significant differences were observed between fed and starved aphids during the initial intervals, but activity levels were similar in the later intervals (Fig. S4D). After 24 and 48 hours of starvation (Fig. S4F and H), there were significant differences between fed and starved aphids in all intervals characterized by hyperactivity of the starved aphids that remained high and resulted in more tactile interactions between the individuals of starved aphids compared to fed aphids (Fig. S5).

4 Discussion

In this study, we first designed and produced a behavioral circular arena to address shortcomings from using Petri dishes. We used this innovative tool to analyze the locomotor activity of freely walking pea aphids and characterize the impact of

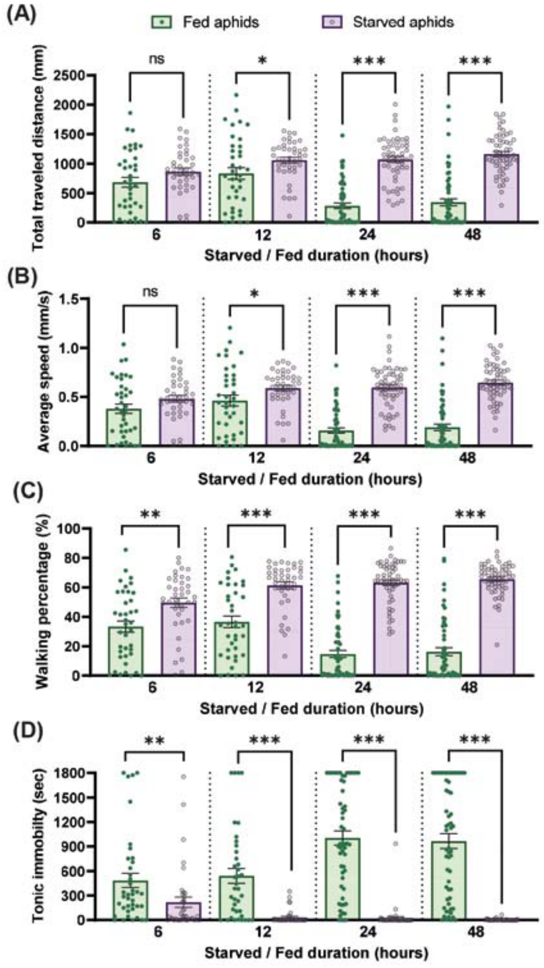

Fig. 4. Starvation-induced locomotor activity of aphids over different starvation durations.
Quantification of total distances traveled **(A)**, average speed **(B),** walking percentage **(C)**, and tonic immobility **(D)** in fed and starved conditions over 30-minute trials. Plots represent mean ± SE, and each dot represents data measurement for one individual. The control for each relative starved group was a similarly conducted experiment without starvation. (Fed-6h, n = 39), (Starved-6h, n = 39), (Fed-12h, n = 38), (Starved-12h, n = 40), (Fed-24h, n = 57), (Starved-24h, n = 54), (Fed-48h, n = 58), and (Starved-48h, n = 57). Mann–Whitney tests were used to compare starved aphids with their parallel-fed aphids. *Asterisks* represent statistical significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant. For statistical details, see Table S1.

extrinsic and intrinsic factors. We found that solitary aphids in large arenas traveled longer distances at higher speed that single aphids in smaller arenas. We discovered that single aphids moved significantly more than aphids in a group. We also found that prolonged periods of starvation resulted in hyperactivity irrespective of length of starvation. Starvation also resulted in a strong reduction and total absence of tonic immobility (TI). TI was not affected by arena size or the number of individuals in the arena. Combined, our data show that space, population size, and nutritional status play crucial roles in the behavioral dynamics of aphids.

Previous studies showed that the size of behavioral arenas used in laboratory experiments impacts animal locomotor activity, but these effects differ between species. For example, fruit flies (Liu et al. 2007), and mice (Kuptsov et al. 2023) displayed increased initial activity levels dependent on arena size, followed by a rapid decrease in the distances traveled over time, while red flour beetles covered larger distances in larger arenas than in smaller ones (Scharf et al. 2024). We found that aphids also increase overall locomotor activity with increasing arena size. These data suggest that aphids adapt their behavior dynamically to the environmental space. Given that single aphids at different arena sizes initially exhibited similar movement levels, we conclude that in larger arenas, aphids increased their step size over time and thus increased traveled distances and speeds upon prolonged stays in the arena. The observation that larger arenas translate into increased speed and larger distances covered is also meaningful in the field since it allows the aphid to rapidly travers open soil to reach a new host plant. Nothing is known of the variables that control this phenomenon. Future studies should investigate the underlying mechanisms of this behavior, including the roles of sensory perception in determining the movement rates.

We found that single aphids traveled significantly longer distances, moved at higher speeds, and spent more time walking than grouped aphids. Previous research also showed that an isolated aphid moves faster when it encounters large nearest-neighbor distances, while it moves slowly with short nearest-neighbor distances (Nilsen et al. 2013). Aphids in groups of 5 and 10, on the other hand, moved the same amount. This observation suggests that social context is crucial in modulating and adjusting locomotor activity. More specifically, this suggests that the presence of conspecifics dampens locomotion, an effect that would be consistent with living in colonies and could depend on visual contact, tactile interactions, and chemical communication, as previously suggested (Couzin-Fuchs & Ayali 2021). Given that aphid density impacts dispersal, this also implies that there may be a threshold above which a dampening effect is no longer observed. Further investigations can not only address whether such threshold exists but could also explore the underlying physiological mechanisms that drive these behavioral differences between solitary and grouped aphids and the implications for population dynamics, social aggregation, and ecological interactions.

Nutritional stress, particularly starvation, is another critical intrinsic factor influencing aphid behavior (Zhang et al. 2019). Our results showed that prolonged starvation profoundly impacted the overall locomotor activity of pea aphids, leading to hyperactivity that was characterized by more frequent movements with long-traveled distances at high speeds. This phenotype is reminiscent of the ‘runner’ phenotype previously reported by Roitberg et al. (1979) that is important to find new host plants further removed from the initial host plant. It has previously been shown that aphids are able to survive off host plants for up to 48 hours while walking on a soil surface between potato plants to find a new host (Alyokhin & Sewell 2003). We observed increased locomotion after 24 and 48 h of starvation but also after 6 and 12 hours. We propose that increased locomotion triggered by hunger after shorter or longer periods of starvation is an intrinsic mechanism ensuring expeditious finding of a new food source. The fact that the increased locomotion after shorter starvation comprises frequent short movements without long travel distances, suggests that aphids explore their surroundings more thoroughly without expending too much energy. Furthermore, we found that starvation causes reduction in tonic immobility after 6 hours and complete absence of TI in all other starvation conditions. These observations suggest that the nutritional deficit induced by starvation results in prioritization of the search for food sources over immobility. Some studies have previously shown that TI is similarly influenced by starvation in other arthropod species such as the snout weevil *Eucryptorrhynchus brandti* (Li et al. 2019), and the ixodid ticks *Dermacentor variabilis* and *Rhipicephalus sanguineus* (Oyen et al. 2021). While we showed that starvation triggers increased locomotor activity in *A. pisum*, further research is needed to investigate the physiological and cellular mechanisms underlying starvation-dependent reductions of TI and starvation-induced hyperactivity.

Our study sets the stage for further exploration into understanding aphid behavioral responses under different experimental conditions, and to characterize behavior and its variation in various aphid species and strains. Strain-associated differences in *A. pisum* have been observed by Kunert et al. (2010) and Zhang et al. (2016) but have not been characterized in detail. We propose that machine learning-based tracking could offer automated monitoring of aphids, allowing the collection of detailed behavioral data on movement patterns (such as relative position, step size, and orientation of aphids), circadian rhythmicity, and interactions with food sources, and responses to predators. The tools described in the current manuscript are readily scalable thereby opening the possibility for (semi-)high-throughput analyses that will not only provide insight in basic mechanisms governing aphid behavior but will ultimately also lead to more effective pest management strategies.

Acknowledgments: We are grateful to Veerle Vulsteke for her assistance with the pea aphid stock culture and to Emily Rosschaert for valuable comments on the manuscript.

References

Alyokhin, A., & Sewell, G. (2003). On-soil movement and plant colonization by walking wingless morphs of three aphid species (Homoptera: Aphididae) in greenhouse arenas. *Environmental Entomology, 32*(6), 1393–1398. https://doi.org/10.1603/0046-225X-32.6.1393

Ben-Ari, M., Gish, M., & Inbar, M. (2015). Walking aphids can partake in within-field dispersal to distant plants. *Basic and Applied Ecology, 16*(2), 162–171. https://doi.org/10.1016/j.baae.2014.11.007

Brodsky, L. M., & Barlow, C. A. (1986). Escape responses of the pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae): Influence of predator type and temperature. *Canadian Journal of Zoology, 64*(4), 937–939. https://doi.org/10.1139/z86-142

Calevro, F., Tagu, D., & Callaerts, P. (2019). Genome of the month: *Acyrthosiphon pisum. Trends in Genetics, 35*(10), 781–782. https://doi.org/10.1016/j.tig.2019.07.003

Chen, J. (2024). Editorial: Aphids as plant pests: from biology to green control technology. *Frontiers in Plant Science, 14*, 1337558. https://doi.org/10.3389/fpls.2023.1337558

Couzin-Fuchs, E., & Ayali, A. (2021). The social brain of non-social insects. *Current Opinion in Insect Science, 48*, 1–7. https://doi.org/10.1016/j.cois.2021.04.006

Diaz, B. M., Barrios, L., & Fereres, A. (2012). Interplant movement and spatial distribution of alate and apterous morphs of *Nasonovia ribisnigri* (Homoptera: Aphididae) on lettuce. *Bulletin of Entomological Research, 102*(4), 406–414. https://doi.org/10.1017/S0007485311000745

Heras, F. J. H., Romero-Ferrero, F., Hinz, R. C., & de Polavieja, G. G. (2019). Deep attention networks reveal the rules of collective motion in zebrafish. *PLoS Computational Biology, 15*(9), e1007354. https://doi.org/10.1371/journal.pcbi.1007354

Hodgson, C. (1991). Dispersal of apterous aphids (Homoptera: Aphididae) from their host plant and its significance. *Bulletin of Entomological Research, 81*(4), 417–427. https://doi.org/10.1017/S0007485300031989

Humphreys, R. K., & Ruxton, G. D. (2018). A review of thanatosis (death feigning) as an anti-predator behaviour. *Behavioral Ecology and Sociobiology, 72*(2), 22. https://doi.org/10.1007/s00265-017-2436-8

IAGC, International Aphid Genomics Consortium. (2010). Genome sequence of the pea aphid *Acyrthosiphon pisum. PLoS Biology, 8*(2), e1000313. https://doi.org/10.1371/journal.pbio.1000313

Irwin, M. E., Kampmeier, G. E., & Weisser, W. W. (2007). Aphid movement: process and consequences. In H. F. van Emden & R. Harrington (Eds.), *Aphids as crop pests* (pp. 153–186). CABI; https://doi.org/10.1079/9780851998190.0153

Kunert, G., Belz, E., Simon, J. C., Weisser, W. W., & Outreman, Y. (2010). Differences in defensive behaviour between host-adapted races of the pea aphid. *Ecological Entomology, 35*(s1, SUPPL. 1), 147–154. https://doi.org/10.1111/j.1365-2311.2009.01146.x

Kuptsov, P. A., Deacon, R. M. J., Anokhin, K. V., & Pleskacheva, M. G. (2023). Exploratory and locomotor activity in mice following selective lesions of the hippocampus: Effects of lesion site and open field arena size. *Journal of Evolutionary Biochemistry and Physiology, 59*(4), 1112–1135. https://doi.org/10.1134/S0022093023040099

Li, H., Zhang, G., Ji, Y., & Wen, J. (2019). Effects of starvation on death-feigning in adult *Eucryptorrhynchus brandti* (Coleoptera: Curculionidae). *Ethology, 125*(9), 645–651. https://doi.org/10.1111/eth.12917

Liu, L., Davis, R. L., & Roman, G. (2007). Exploratory activity in *Drosophila* requires the kurtz nonvisual arrestin. *Genetics, 175*(3), 1197–1212. https://doi.org/10.1534/genetics.106.068411

Luxem, K., Sun, J. J., Bradley, S. P., Krishnan, K., Yttri, E., Zimmermann, J., … Laubach, M. (2023). Open-source tools for behavioral video analysis: Setup, methods, and best practices. *eLife, 12*, e79305. https://doi.org/10.7554/eLife.79305

Müller, C. B., Williams, I. S., & Hardie, J. (2001). The role of nutrition, crowding and interspecific interactions in the development of winged aphids. *Ecological Entomology, 26*(3), 330–340. https://doi.org/10.1046/j.1365-2311.2001.00321.x

Nilsen, C., Paige, J., Warner, O., Mayhew, B., Sutley, R., Lam, M., … Topaz, C. M. (2013). Social Aggregation in Pea Aphids: Experiment and Random Walk Modeling. *PLoS One, 8*(12), e83343. https://doi.org/10.1371/journal.pone.0083343

Oyen, K. J., Croucher, L., & Benoit, J. B. (2021). Tonic immobility is influenced by starvation, life stage, and body mass in Ixodid ticks. *Journal of Medical Entomology, 58*(3), 1030–1040. https://doi.org/10.1093/jme/tjab003

Ribeiro Lopes, M., Gaget, K., Renoz, F., Duport, G., Balmand, S., Charles, H., … Calevro, F. (2022). Bacteriocyte plasticity in pea aphids facing amino acid stress or starvation during development. *Frontiers in Physiology, 13*, 982920. https://doi.org/10.3389/fphys.2022.982920

Roitberg, B. D., & Myers, J. H. (1979). Behavioural and physiological adaptations of pea aphids (Homoptera: Aphididae) to high ground temperatures and predator disturbance. *Canadian Entomologist, 111*(4), 515–519. https://doi.org/10.4039/Ent111515-4

Roitberg, B. D., Myers, J. H., & Frazer, B. D. (1979). The influence of predators on the movement of apterous pea aphids between plants. *Journal of Animal Ecology, 48*(1), 111–122. https://doi.org/10.2307/4104

Romero-Ferrero, F., Bergomi, M. G., Hinz, R. C., Heras, F. J. H., & de Polavieja, G. G. (2019). idtracker.ai: Tracking all individuals in small or large collectives of unmarked animals. *Nature Methods, 16*(2), 179–182. https://doi.org/10.1038/s41592-018-0295-5

Sapountzis, P., Duport, G., Balmand, S., Gaget, K., Jaubert-Possamai, S., Febvay, G., … Calevro, F. (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 Biochemistry and Molecular Biology, 51*(1), 20–32. https://doi.org/10.1016/j.ibmb.2014.05.005

Scharf, I., Hanna, K., & Gottlieb, D. (2024). Experimental arena settings might lead to misinterpretation of movement properties. *Insect Science, 31*(1), 271–284. https://doi.org/10.1111/1744-7917.13213

Sochard, C., Leclair, M., Simon, J.-C., & Outreman, Y. (2019). Host plant effects on the outcomes of defensive symbioses in the pea aphid complex. *Evolutionary Ecology, 33*(5), 651–669. https://doi.org/10.1007/s10682-019-10005-4

van Emden, H. F., & Harrington, R. (Eds.). (2007). *Aphids as crop pests*. CABI; https://doi.org/10.1079/9780851998190.0000

Zhang, D.-W., Xiao, Z.-J., Zeng, B.-P., Li, K., & Tang, Y.-L. (2019). Insect behavior and physiological adaptation mechanisms under starvation stress. *Frontiers in Physiology, 10*, 163. https://doi.org/10.3389/fphys.2019.00163

Zhang, Y., Wang, X.-X., Zhu, J.-Y., Zhang, Z.-F., Tian, H.-G., & Liu, T.-X. (2016). Strategies used by two apterous strains of the pea aphid *Acyrthosiphon pisum* for passive dispersal. *Biology Open, 5*(10), 1535–1544. https://doi.org/10.1242/bio.018903

Manuscript received: July 24, 2024
Revisions requested: October 26, 2024
Revised version received: January 13, 2025
Manuscript accepted: January 28, 2025

The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement:
Fig. S1–S5, Table S1, S2