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Assessing learning in mosquito larvae using video-tracking

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ABSTRACT

Mosquito larvae display a stereotyped escape response when they rest attached to the water surface. It consists in detaching from the surface and diving, to return to the surface after a brief time. It has been shown that this response can be evoked several times, by repeatedly presenting a moving shadow. Diving triggered by a potential danger revealed as a simple bioassay for investigating behavioural responses in mosquito larvae, in particular their ability to learn. In the present work, we describe an automated system, based on video-tracking individuals, and extracting quantitative data of their movements. We validated our system, by reinvestigating the habituation response of larvae of *Aedes aegypti* reared in the laboratory, and providing original data on field-collected larvae of genera *Culex* and *Anopheles*. Habituation could be demonstrated to occur in all the species, even though it was not possible to induce dishabituation in *Culex* and *Anopheles* mosquitoes. In addition to non-associative learning, we characterised motor activity in the studied species, thanks to the possibility offered by the tracking system to extract multiple variables. The here-described system and algorithms can be easily adapted to multiple experimental situations and variables of interest.

1. Introduction

Adapting individual behaviour on the basis of the own experience (i.e. learning) and remember past experiences (i.e. memory) is crucial for an animal to survive and to make decisions (Evans et al., 2019).

Habituation is a particular form of non-associative learning (Thomas, 1949; Leftwich, 1954) which consists in no longer reacting to stimuli that trigger behavioural response in naïve animals and turned out to be innocuous (See Rankin et al., 2009 for review). For example, when a moving object casts its shadow over the water surface, mosquito larvae dive escaping from a potential danger (Holmes, 1911). After several passages of an innocuous shadow, larvae stop responding, even though they still detect it and they are able to dive. The individuals do not perceive the stimulus as a potential danger anymore, i.e. the larvae become habituated to its presence (Baglan et al., 2017).

Habituation protocols in mosquito larvae have revealed to be reliable bioassays, not only for testing cognitive abilities of these insects (e.g. Baglan et al., 2017; Pientrantuono et al., 2021), but also as a proxy for evaluating the impact on living creatures of chemical pollutants in water (Baglan et al., 2018). In a typical experiment, an observer records whether or not individual larvae move during the controlled passage of a moving shadow, attributing a score of 0 or 1. The shadow is presented at regular intervals, until the insects stop responding. Specific tests follow, in order to assess whether the behavioural change is either due to learning or to other physiological processes, such as sensory adaptation or motor fatigue (for a review, see Rankin et al., 2009).

In this work, we present an original system allowing the automated quantification of different components of the response of mosquito larvae to a potential danger. Our tracking software allows accurately measuring individual response, and calculating different metrics associated with diverse components of the behavioural response, minimising experimental biases.

The system allows training and testing several individuals in parallel in a single session, saving time, increasing the number of replicates, and obtaining accurate quantitative data on different behavioural variables. Training parameters as intensity and duration of the stimulus, inter-trial interval, interval between training and test can be precisely adjusted by the experimenter. We started by testing and validating our system and the experimental protocol for habituation experiments in a reference species (i.e. *Aedes aegypti*) and then we compared the responses among laboratory and field-collected mosquito larvae of other species.

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2. Material and methods

2.1. Animals

Aedes aegypti (Bora strain) were obtained from eggs provided by the INFRAVEC2 European project and reared at VECTOPOLE-IRD (Montpellier, France). The eggs were placed in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). The larvae were maintained in a climate-controlled room at 25 °C ± 2 °C, under 12 h:12 h light:dark illumination regime (lights on at 8:00).

Culex and *Anopheles* larvae were collected in two natural habitats located in the department of Indre et Loire, France. The first site was a 10-ha basin (*Étang de l' Archevêque*) located in the Loire Valley (47°31′N, 0°51′E), and the second was a pond situated in an urban garden in the city of Tours (47°23′N, 0°41′E). At each site, captures were carried out over a sampling area of approximately 1 m^2 by scooping the surface with a 1-litre round recipient (O'Malley, 1995), until at least 100 individuals were collected. Sampled individuals were kept in water from their natural habitat during the journey back to the laboratory and then gently transferred to 750 ml polypropylene plastic containers and reared similarly as *Ae. aegypti* larvae. Before experiments, individuals were kept undisturbed between 24 h and 48 h under laboratory conditions (the same for all species). Fourth-instar larvae were used in all the experiments.

All animals were collected, reared and manipulated according to ethics regulations applied in the European Union.

2.2. Identification

For *Culex* and *Anopheles*, the morphological identification of individuals was performed under a stereomicroscope, with the aid of the MosKeyTool database (Gunay et al. 2018). An initial identification was conducted after the experiment to assess the genus at larval stage. Individuals were kept until emergence, then adults were identified a second time to determine sex and confirm genus, using the same key.

We found at least two species of *Culex* using the Moskeytool database: *Culex pipiens* and *Culex territans*. We were able to distinguish these two species by comparing their siphon index (total length/diameter at the base) and the arrangement of their siphon setae. For the genus *Anopheles*, it was much more difficult to determine the species, but it is most likely that the individuals belonged to the *Anopheles maculipennis* complex. The Moskeytool database did not go further than this complex and we could not find any visible differences between the *Anopheles* individuals. We also identified the genus *Culiseta*, but we excluded these larvae, because they were rarely found in our samples.

2.3. Experimental setup

The experimental apparatus (Fig. 1), consisted of two light sources, a camera, and a servo mechanism, which controlled the projection of the shadow of a square cardboard (*shadow*) above twelve 1.5 ml spectrophotometer plastic cuvettes (Z187992-1PAK, Sigma-Aldrich, Germany) where the larvae had been individually placed. One light source consisted of two LED panels (30 cm × 30 cm), located above the cuvettes. The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with a high-pass infrared filter (RG 850 Filter – 40.5 mm, Heliopan, US) recorded the experiments.

In order to exclude unwanted vibrations, cuvettes stood on a 2 cm-thick polystyrene plate resting over acoustic foam. The lateral faces of each cuvette were covered with opaque white tape, in order to avoid mutual visual influence. A water-filled cuvette without larvae was placed at the left of the 1st cuvette and another one at the right of the 10th cuvette, to minimise any effect of cuvette position.

Two stimuli of different modality could be presented. The first (visual) was the shadow projected by a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board (https://www.arduino.cc). During a stimulation, the stick turned 100° and returned back to the resting position (Fig. 1). The second stimulus (mechanical) was the vibration produced by a set of 4 identical vibrators (3.3 V, 100 mA; 11000 rpm; 10 mm diameter; 2.7 mm height, Radio Spares, France), controlled by the same Arduino Uno board. Two vibrators were placed on the outer side of the left end and

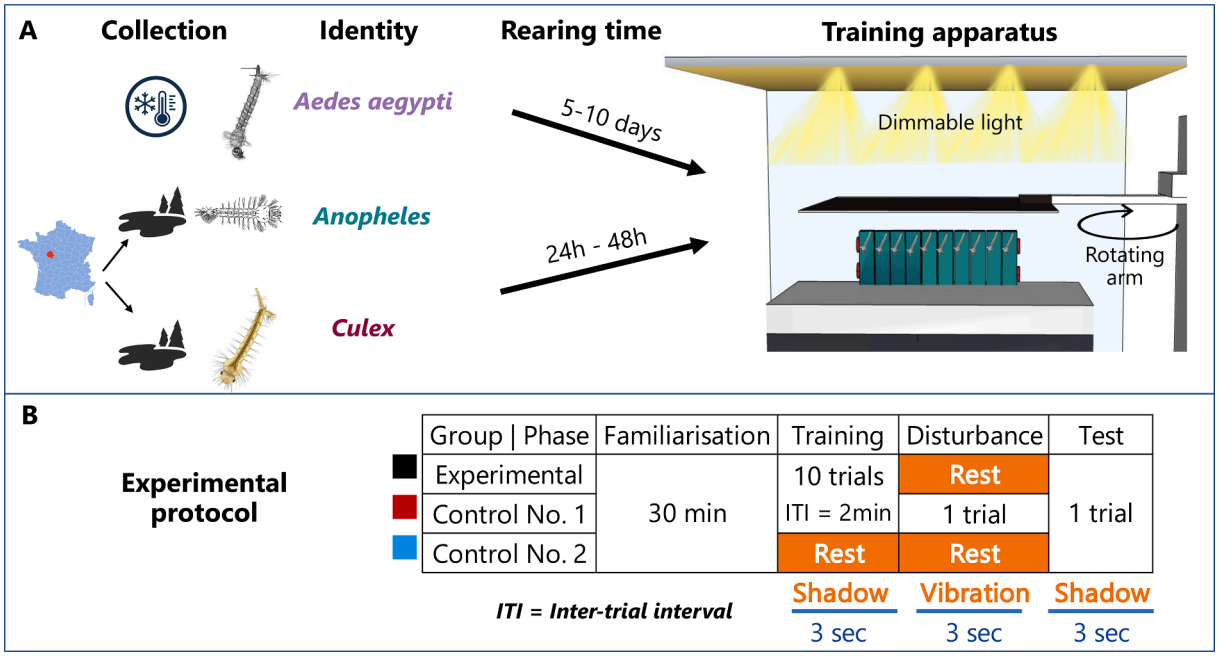

Fig. 1. Experimental protocol. A) We collected *Culex* and *Anopheles* larvae in two ponds located in Indre-et-Loire, Région Centre-Val de Loire, whilst *Aedes aegypti* larvae were reared in the laboratory. We trained individuals of the 4th larval stage using an automated device. B) We quantified the responses of three groups: 1) trained and not disturbed (Experimental); 2) trained and disturbed (*Control No. 1*); 3) untrained (*Control No.* 2).

the other two at the right end, i.e., on the side of the unoccupied cuvettes. The Arduino board was remotely controlled by a computer, which was also connected to the camera.

Preliminary tests revealed differences in the responsiveness of the larvae belonging to the different species. For this reason, a series of experiences were run in order to establish the appropriate parameters for testing each species.

For *Ae. aegypti* light intensity was set at 900 μW.cm^{-2} ± 100 μW.cm^{-2} (International Light Technology radiometer). The distance between the top of the cuvettes and the rotating arm was established in 5 ± 0.2 cm and the stimulus duration fixed at 3 s at an angular velocity of 0.067°/ms.

For *Culex* and *Anopheles* larvae, we increased the light intensity to 1500 μW.cm^{-2} ± 100 μW.cm^{-2} and placed the card closer to the top of the cuvette (0.3 ± 0.1 cm). We also increased the arm rotation to 7.5°/ms and added 1.5 s of delay in the stimulus position, to keep the total stimulus duration at 3 s. The goal was ensuring that most of the larvae would react.

2.4. Experimental conditions

All experiments were performed in a room kept at the rearing temperature. Larvae were carefully removed from the rearing container and placed individually in the cuvettes filled with dechlorinated tap water.

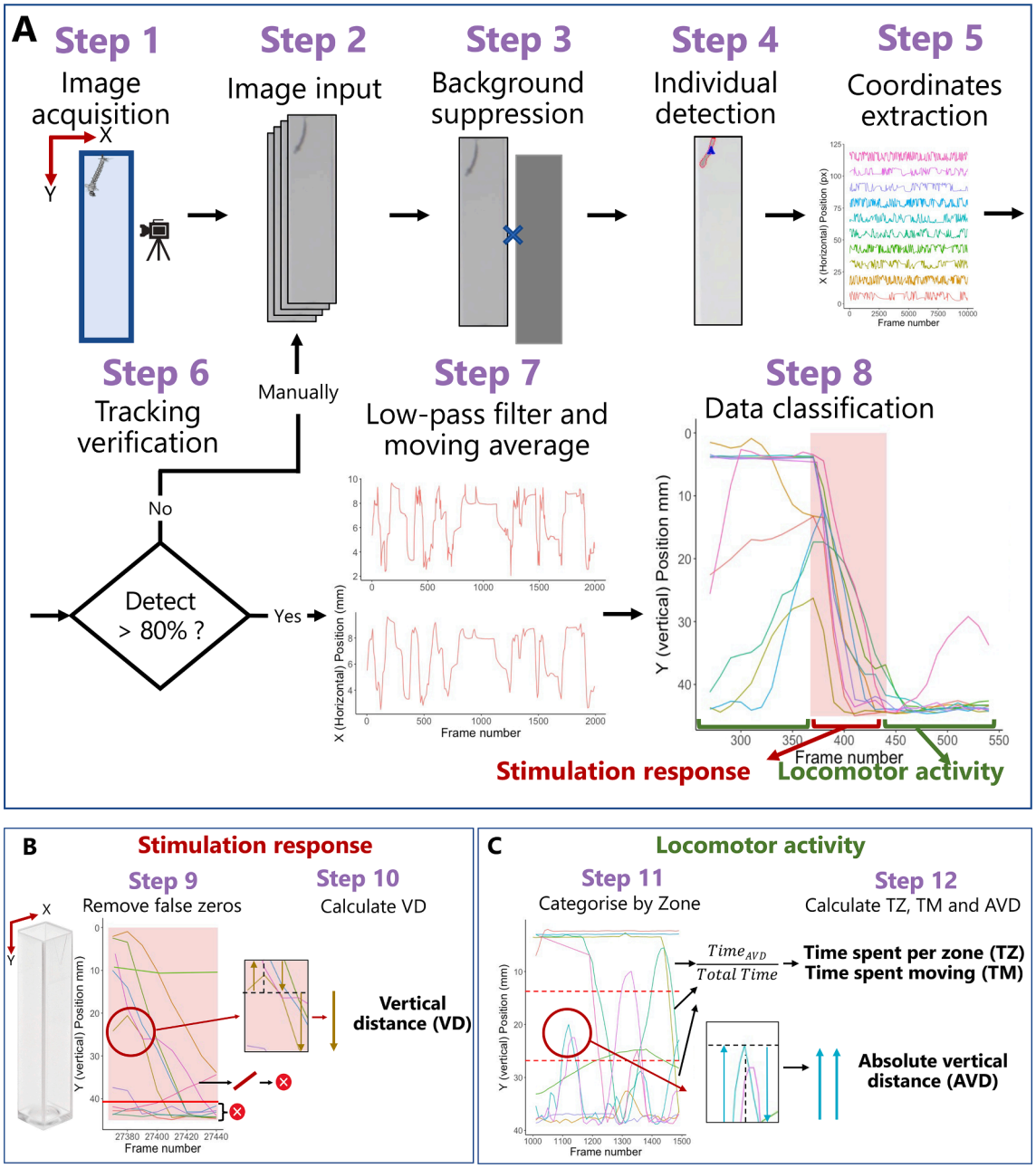

Fig. 2. Recording and quantifying individual behaviour. A) Flowchart of data acquisition and treatment. B) Response to visual stimulus (SR) was analysed using two metrics. Performance Index (PI) was binary and calculated following the trajectory direction of each individual for each trial. Vertical distance (VD) was quantitative and calculated as the relative sum of the distance travelled in the vertical direction. C) Locomotor activity (LA) was analysed using three metrics. Time spent per zone (TZ) was a proportion of time spend in one of the 3 zones delimited. Time spent moving (TM) was a proportion of time where the Absolute vertical distance was above a threshold of 1 mm/sec. Absolute vertical distance (AVD) was quantitative and calculated as the absolute sum of the distance travelled in the vertical direction.

Larvae were left undisturbed during 30 min for familiarisation before starting the experiment. Under these conditions, we established one experimental group and two control groups. The procedure consisted of three phases: *training or rest*, *disturbance or rest* and *test* (Fig. 1).

The *Experimental* group was set to assess the decrease in response induced by the repeated presentation of the visual stimulus (*training*). Larvae were confronted with 10 passages of the shadow (i.e. *trials*), spaced by inter-trial intervals (ITI) of 2 min. After the 10th presentation of the stimulus, larvae remained undisturbed during 4 min, before the final presentation of the shadow; i.e., the *test* phase.

Control No. 1 (disturbance) was set to assess dishabituation (Rankin et al., 2009). Larvae were exposed to 10 stimuli, similarly to the experimental group (*training*). Two minutes after the 10th stimulus, a vibration was applied (*disturbance*). This disturbance was followed by 2 min ITI and the final presentation of the shadow (*test*).

Control No. 2 (untrained), after familiarisation, larvae remained confined in the cuvettes during 22 min without receiving any stimulation. Subsequently, the visual stimulus was presented to the larvae only in the *test* phase.

After the end of each experiment, larvae were gently removed from the cuvettes and individually kept in identified Petri dishes (3-cm diameter) during 24 h. Those that emerged as adults during this time could have been pharate pupae during the experiment and consequently excluded from the analyses.

2.5. Video analyses

Each experiment was recorded and two sets of videos (resolution 640x480 px, 25 fps) were produced (Fig. 2, step 1). The first one consisted of sequences of the last 5 min of familiarisation. The second set consisted of 26 min videos of the three phases of each experiment (i.e., *training*, *disturbance* and *test*). The videos were analysed using a modified version of the image-based freeware Tracktor (Sridhar et al., 2019).

The tracking software was based on a contour identification algorithm relying on the contrast between the larvae and the background. (Fig. 2, step 2 and 3). During the video analysis, the position and contour area of each larva were measured while keeping identity (Fig. 2, step 4). At the end of the video analysis measurements were exported to a.*csv* file.

The tracking results were analysed using R version 4.1.1 (2021-08-10) (https://cran.r-project.org/). Horizontal coordinates were used to verify that larvae identities were respected (each larva detected inside a given cuvette, Fig. 2, step 5). To check the performance of the tracking, the detection rate was calculated by taking the maximum frame length available on the video and multiplying it by the number of individuals. This rate was compared to the actual number of frames identified by the tracking software and we ensured that at least 80% of the data present (Fig. 2, step 6). The vertical position data were smoothed using the *rollmean* function in the *zoo* package (Zeileis and Grothendieck, 2005) with a 10-frame window (Fig. 2, step 7).

2.6. Data classification and analysis

From each dataset and each trial, we defined and extracted the 3 s trial period as the duration of the stimulus appearance over the individuals (Fig. 2, step 8). Therefore, we could extract 8 successive positions for each individual and for each trial that were classified as Stimulation Response dataset (Fig. 2).

No vertical displacement could be observed in the larvae that were at the bottom of the cuvette at the beginning of a trial. Therefore, we excluded the response of individuals whose vertical position at the start of a trial was higher than 9/10th of the cuvette length (i.e. close to the bottom) (Fig. 2, step 9).

Vertical distance (VD) was the response variable and corresponded to the escape response, starting from 0 at the top of the cuvette, and increasing, when larvae dived along the water column (Fig. 2, step 10). We also defined a binary criterion as:

Therefore, we calculated the proportion of individuals (i.e., Performance Index, PI) that dived enough to be considered responding.

For *Culex* and *Anopheles* species, some individuals rested completely motionless during one trial. For this trial, the value given for their displacement was therefore counted as 0 mm for VD and for PI. When one individual was completely immobile during the acclimation and the training period, it was removed from the database (8.5% for *Anopheles*, 0% for others). A total of 246 individuals were retained for the analysis (Table 1).

As individual positions were recorded throughout the whole experiments, we also extracted data during the 9 inter-trial intervals (ITIs) and analysed the locomotor activity during these periods (Fig. 2). We first calculated the Absolute Vertical Distance (AVD) travelled by individuals by summing the AVD for all ITIs per individual during the training session (Fig. 2, step 11). We also ranked data by ITI and compared the AVD per ITI for each species. The AVD was then averaged per second and calculated for each individual to compare the individual average speed during the ITIs. The maximum speed of each individual was also compared in the same way.

We divided the cuvette in three equal zones (top, middle, bottom, Fig. 2, step 12) and calculated the time spent per zone. We used these zones to develop another metric corresponding to the diving events. If an individual crossed two successive zones on the way in and out, we considered this to be a diving event. To analyse if an individual was moving or not based on a dichotomous rule, we also confronted the individual AVD to a threshold of 1 mm per second and classified the resulting data in Time spent moving (Fig. 2, step 12). Prior to any training, individuals were recorded for 30 min during the familiarisation period. To highlight the effect of the stimulation on individual activity, we analysed the last 5 min of familiarisation and compared them to the ITI periods. Finally, using contour tracking data, we were able to compare the maximum individual surface detected by the tracking between species, i.e., the area representing each individual in pixel.

3. Statistical analyses

3.1. Data classification and filtering

For the three species, we verified whether responses to stimulation were trial-specific (i.e. increased or decreased) by applying a Chi-square goodness of fit test. The rationale behind this verification was to exclude that larvae could have changed their position before the release of the stimulus over the course of the training, then biasing the output of the filtering.

3.2. Power analysis

Using the *"simr"* package in R (Green and MacLeod, 2016), we performed a power analysis to confirm the power of our sample size. For the Vertical Distance variable and for each species, we used the function *powerSim*. For 1000 simulations and alpha = 0.05, the power was 95.90% CI [94.48, 97.04] for *Aedes aegypti*, 89.50% [87.43, 91.33] for *Culex* and 98.70% [97.79, 99.31] for *Anopheles*.

3.3. Comparison across species

For the three mosquitoes, we used a Generalised Additive Model to explore different response curves for each variable and their effect on the generalised cross-validation unbiased risk estimator (GCV-UBRE) (Zuur et al., 2009). We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE of the *mgcv* package (Wood, 2017).

To compare locomotor activity between species, we used linear mix-effects models. These models were used for the comparison of AVD (m), average speed (mm/s) and maximum speed (mm/s), time spent per zone

Table 1
Summary of the filtering steps. For each species, 22 to 30 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 13.9% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 3.4% of trials.

	Aedes aegypti				*Culex*				*Anopheles*				All
	Experimental	Control n°1	Control n°2	Total	Experimental	Control n°1	Control n°2	Total	Experimental	Control n°1	Control n°2	Total	Total
Individuals trained	30	30	30	90	26	29	27	82	22	28	24	74	246
Trials per individuals	360	360	30	750	310	347	27	684	191	313	24	528	1962
Trials filtered by position	274	268	24	566	271	312	27	610	189	301	23	513	1689
% Trials removed	23.9%	25.6%	20.0%	24.5%	12.6%	10.1%	0.0%	10.8%	1.0%	3.8%	4.2%	2.8%	13.9%
Trials filtered by going up	268	263	24	555	261	290	26	577	184	293	23	500	1632
% Trials removed	2.2%	1.9%	0.0%	1.9%	3.7%	7.1%	3.7%	5.4%	2.6%	2.7%	0.0%	2.5%	3.4%
Total % Trials removed	25.6%	26.9%	20.0%	26.0%	15.8%	16.4%	3.7%	15.6%	3.7%	6.4%	4.2%	5.3%	16.8%

(%) and time spent moving (%), number of diving event and maximum surface (mm^2). We chose species as a fixed factor and individual identity as a random factor. Post-hoc comparisons were analysed using the *emmeans* function from the *emmeans* package (Russell, 2021).

3.4. Learning performance

To assess the learning performances of the different groups of larvae, we compared Vertical Distance and Performance Index. VD was evaluated by means of a linear mix-effects model and PI of a GLMER with a logit link and a binomial distribution, with trial number and group as fixed factors, and individual identity as random factor to account for repeated measurements. The group factor served to evaluate eventual effects due to contexts across groups of larvae trained in a similar way. As the interaction between trial and group was never significant, we dropped the interaction from the model.

3.5. Test phase

Test responses were analysed by running a linear mix-effects model for VD and a GLMER for PI with a logit link and a binomial distribution. VD and PI were chosen as the response variables; group as fixed factor, and individual identity as random factor.

3.6. Dishabituation

To assess dishabituation, we compared VD and PI at the tenth trial with the response at the *disturbance* and at the *test* phase. VD was compared by using a linear mix-effects model and PI by means of GLMER, similarly as in the section Learning performance.

3.7. Dataset and analysis code repository

The version of the tracking software used to characterise individual behaviour and the R code used to analyse data and display the figures were made available online at: https://github.com/martindessart/Tracking-system.

4. Results

4.1. Identification

Larvae and adults could be identified at the genus level. For *Anopheles,* we were able to evaluate the sex of 25 individuals out of the 74 total individuals trained, of which 13 were identified as females and 12 as males. For *Culex,* 40 individuals were identified as females and 24 as males.

4.2. Data classification and filtering

At 25 frames per second, a 22-minutes recording corresponded to a total of 33 000 frames. Our tracking algorithm, adapted from the open source software Tracktor (Sridhar et al., 2019) had a tracking time of<20 min. For comparison, the zebrafish video from ToxTrac software (Rodriguez et al., 2018), with a resolution of 32 frames per second and 15 000 frames, had a tracking time of 9 min 43 s using Tracktor software (Sridhar et al., 2019). Regarding accuracy, the total percentage of detection rate was 92.54% for 22 records. The highest detection rate was 99.97% and the lowest 84.84%. For each experiment, we performed a calibration by zooming in on the cuvettes with our camera and applying the function "Automatic image adjustment" from Basler Pylon5 software (https://baslerweb.com). Provided that each cuvette was physically separated from the others, a handmade function on R software using the total horizontal distance (*x*-coordinate) divided by the number of individuals allowed us to identify all individuals for all videos. Finally, for each recording, we manually selected the square outline of the 10

cuvettes. Then, for each species, we took the mean of the maximum distance in pixels for each record and converted the pixel unit to millimetres. The mean distance was: Mean 401.4 px, SD = ±9.01 px.

Our tracking system was able to discriminate individuals based on their vertical position at the start of each stimulation. When an individual was above a threshold of 9/10th of the total length of the cuvette, the filtering step (Fig. 2, step 9) eliminated an average of 13.9% of the trials for all species (Table 1). This percentage depended on the species, with 24.5% for *Ae. aegypti,* 10.8% for *Culex* and 3.0% for *Anopheles* Furthermore, the binary criterion detected and eliminated an additional 3.3% of responses to trials where individual VD was greater than 1/5th of the maximum VD. This was more pronounced for *Culex* (5.4% of removed trials) than for *Anopheles* (2.4%) and *Ae. aegypti* (1.9%). The overall process resulted in 1632 stimulation responses for 246 individuals. To determine whether the trials deleted in these two successive steps were trial-specific, we confronted this hypothesis using a Chi-square goodness of fit test. The deleted trials were not specific to a trial number for the three species: *Ae. aegypti*: $X^2 = 10.81$, df = 11, P = 0.459; *Anopheles:*$X^2 = 8.11$, df = 11, P = 0.703; *Culex*: $X^2 = 15.59$, df = 11, P = 0.157.

The performance of our automated system was compared to human visual characterisation. Three different experimenters scored manually the behavioural response of *Aedes aegypti* by looking at the videos. Overall, the confidence intervals were larger for the visual classification (Experimenter #1: 95% CI [0.94, 1.06], Experimenter #2: 95% CI [0.92, 1.08], Experimenter #3: 95% CI [0.93, 1.07]) than for the automated approach (95% CI [0.95, 1.04]). In addition, there were significant differences in scores between the three experimenters. The percentage of unequally scored data between Experimenter #1 and Experimenter #2 was 34.4%; Experimenter #1 and Experimenter #3 was 26.2% and Experimenter #2 and Experimenter #3 was 32.5%. Overall, the percentage similarity between the three experimenters was 53.4%. So, visual categorisation revealed less precise than the automated one.

4.3. General comparison across species

The response of the larvae decreased with the consecutive passage of the shadow. In other words, the escape response was less intense over the course of the ten trials, both for PI and VD (Fig. 3A, B). Concerning PI, a difference in the number of responsive larvae (Escape response = 1) was observed across species, being *Ae. aegypti* the most responsive and *Anopheles* the least. All three decreased across trials at similar rates, curves running parallel at different levels.

To describe the variation in VD, the best smoothing function was the P-spline, as it is based on equally spaced knots (Wood, 2017). Plotting the mean distance (mm) against the number of trials for the three species revealed different responses (Fig. 3B). *Ae. aegypti* responded strongly to the stimulus (Mean = 22.69, SEM = ±0.63), *Culex* was weaker than *Ae. aegypti* (Mean = 16.09, SEM = ±1.11) and *Anopheles* responded the least (Mean = 6.50, SEM = ±0.1) (Fig. 3A, B). *Anopheles* also decreased their response more steeply than *Ae. aegypti* and *Culex* (Fig. 3A, B).

Concerning spontaneous locomotor activity, *Ae. aegypti* and *Culex* moved significantly more than *Anopheles* (P < 0.0001 in both cases) but did not differ from each other (P = 0.758); see supplementary Fig. S1A. Regarding within-trial differences, the three species did not show significant differences among trials (*Ae. aegypti*: df = 8, P = 0.990, *Culex:* df = 8, P = 0.999, *Anopheles*: df = 8, P = 0.100); see supplementary Fig. S1B. Regarding the average speed, while *Ae. aegypti* and *Culex* were significantly faster than *Anopheles* (P < 0.0001 in both cases, Supplementary Fig. 2A), the latter reached higher maximum speed than *Ae. aegypti* and *Culex* (P < 0.0001 in both cases). *Culex* had a higher maximum speed than *Ae. aegypti* (P < 0.0001) but was not faster (P = 0.488); see supplementary Fig. S2A, B. Similarly, *Anopheles* spent little time moving (ca. 11% of the time), while *Ae. aegypti* was very active (ca. 80% of the time) and *Culex* was moderately active (ca. 47% of the time); see supplementary Fig. S3B). While *Anopheles* spent more than 75% of its time near the surface, *Culex* spent more than 25% in the middle and at the bottom zone of the cuvette and *Ae. aegypti* spent more time at the bottom zone; see supplementary Fig. S3A. The difference in activity was maintained when comparing the number of diving event with *Ae. aegypti* and *Culex* diving more than *Anopheles* (both P < 0.0001), but there was no difference between *Ae. aegypti* and *Culex* (P = 0.690): see supplementary Fig. S4A. On average, *Ae. aegypti* and *Anopheles* images had similar surface area (in pixels) (P = 0.438) and were larger than *Culex* (both P < 0.0001); supplementary Fig. S4B).

Finally, movement comparisons between familiarisation and ITI for *Ae. aegypti* showed no difference in average speed (P = 0.834); see supplementary Fig. S5A), but a significant difference in maximum speed (P < 0.0001); supplementary Fig. S5B). The comparison of time spent moving show little difference (P = 0.046); supplementary Fig. S5D.

4.4. Training phase

Learning performance was assessed by comparing individual responses between the 1st and the 10th trials (Fig. 3). For the three species, these comparisons rendered significant differences, evincing a decrease

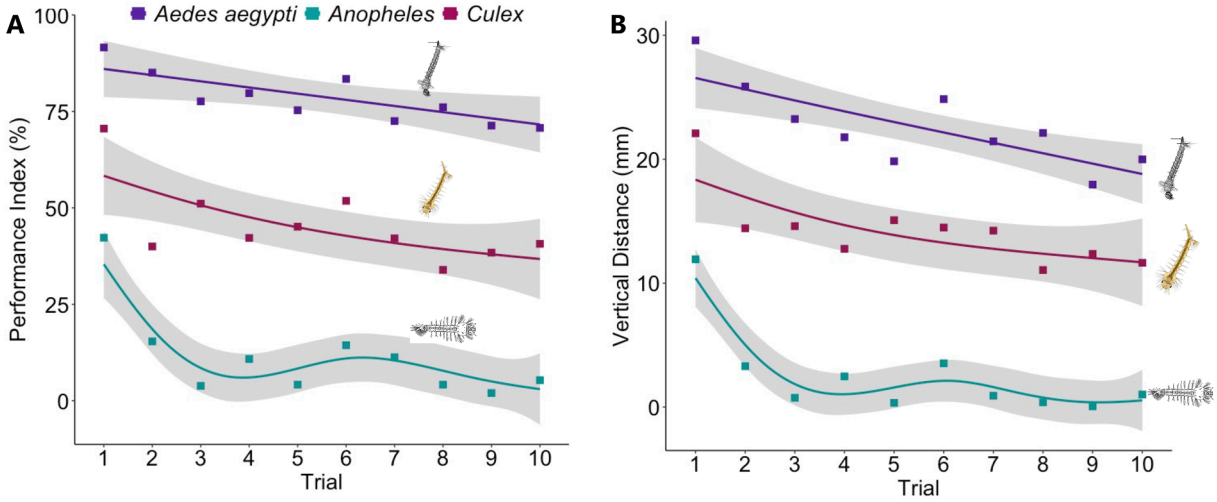

Fig. 3. Behavioural response over the course of the training phase. A) Performance Index of individuals responding to the visual stimulus in each *training* trial. B) Vertical distance in millimetres travelled by individuals responding to the visual stimulus from the 1st till the 10th *training* trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval. Points indicate mean values.

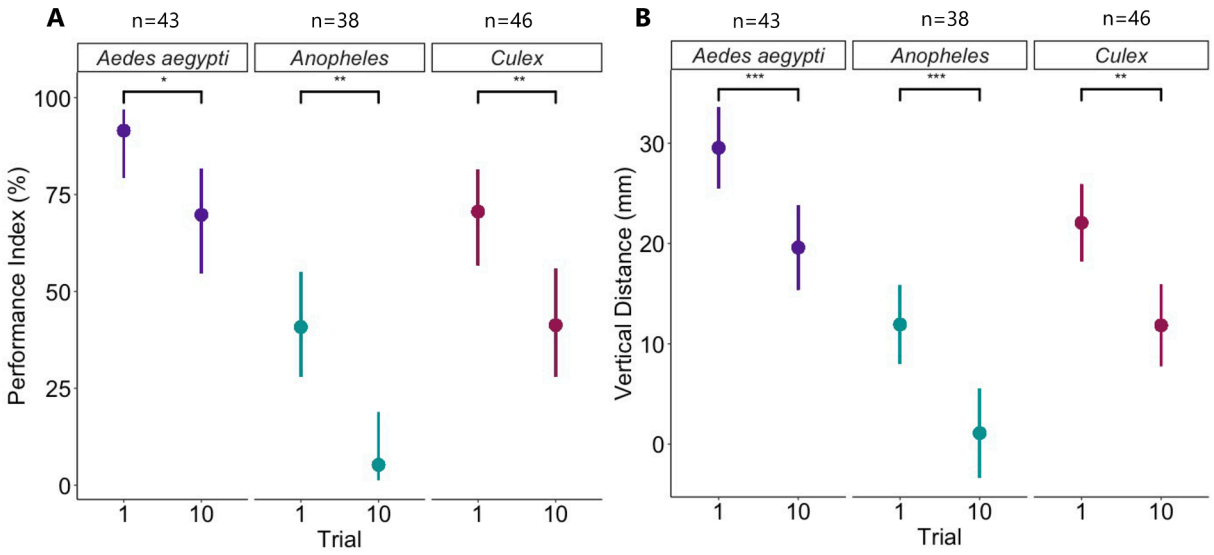

Fig. 4. Learning performance. A) Performance Index of individuals responding to the visual stimulus in the 1st trial and in the 10th trial. B) Vertical distance travelled by individuals responding to the visual stimulus in the 1st trial and in the 10th trial of the *training* phase. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

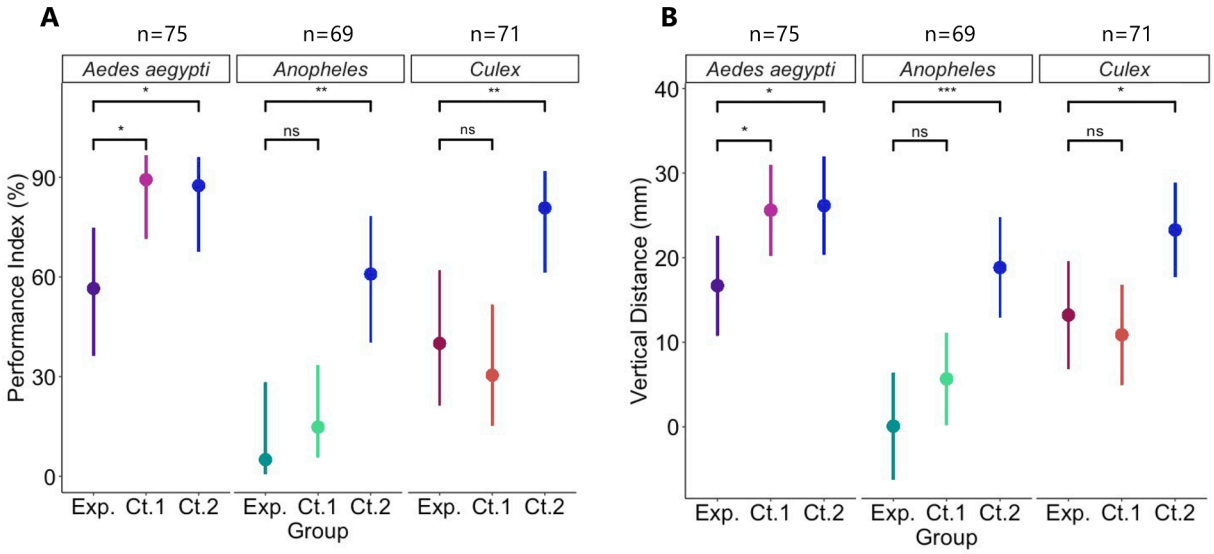

Fig. 5. Test phase. A) Performance Index of individuals responding to the visual stimulus during the *test* phase (i.e. after the *training* phase). B) Vertical distance travelled by individuals responding to the visual stimulus during the *test* phase. Dark purple, dark green and dark red indicate *experimental* group (Exp.) for each species. Light purple, light green and light red indicate *Control No. 1* (Ct.1) for each species. Blue indicates *Control No. 2* (Ct.2) for each species. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in responsiveness (Fig. 4). The Performance index was higher at the 1st than at the 10th trial (*Ae. aegypti:* $X^2 = 5.93$, df = 1, P = 0.015; *Anopheles:* $X^2 = 9.17$, df = 1, P < 0.01; *Culex:* $X^2 = 8.01$, df = 1, P < 0.01) (Fig. 4A). Vertical distance (mm) was also higher at the 1st than at the 10th trial (*Ae. aegypti:* $X^2 = 13.786$, df = 1, P < 0.001; *Anopheles:* $X^2 = 17.957$, df = 1, P < 0.001; *Culex:* $X^2 = 10.472$, df = 1, P < 0.01) (Fig. 4B).

4.5. Habituation assessment

For each species, we compared the response at the *test* trial on PI and VD between *Experimental* group, *Control No. 1* and *Control No. 2* (Figs. 5, 6). For *Ae. aegypti*, the response level of the *Experimental* group was significantly lower than in *Control No. 1* (PI: 95% CI [0.40, 4.02], P = 0.016; VD: $t_{70} = 2.53$, P = 0.014) and in *Control No. 2* (PI: 95% CI [0.21, 3.92], P = 0.029; VD: $t_{70} = 2.66$, P = 0.010, Fig. 6). For *Culex* and *Anopheles*, the response of the *Experimental* group was significantly lower than that of *Control No. 2* (*Culex* PI: 95% CI [0.48, 3.32], P = 0.009; VD: $t_{64} = 2.07$, P = 0.042; *Anopheles* PI: 95% CI [1.17, 6.34], P = 0.004; VD: $t_{66} = 4.00$, P < 0.001) but not relative to *Control No. 1* (*Culex* PI: 95% CI [-1.75, 0.86], P = 0.507; $t_{63} = 0.47$, P = 0.643; *Anopheles* PI: 95% CI [-1.23, 3.49], P = 0.347; VD: $t_{66} = 1.38$, P = 0.173), concerning both PI and VD (Fig. 5).

By comparing individual response between the 10th *training* trial and the *test* phase (i.e, after the disturbance), we looked for evidence of dishabituation to occur. Both, the PI and VD showed contrasted performance for dishabituation across species. *Ae. aegypti* was the only out of the three mosquitoes analysed to show a reversal of the habituation

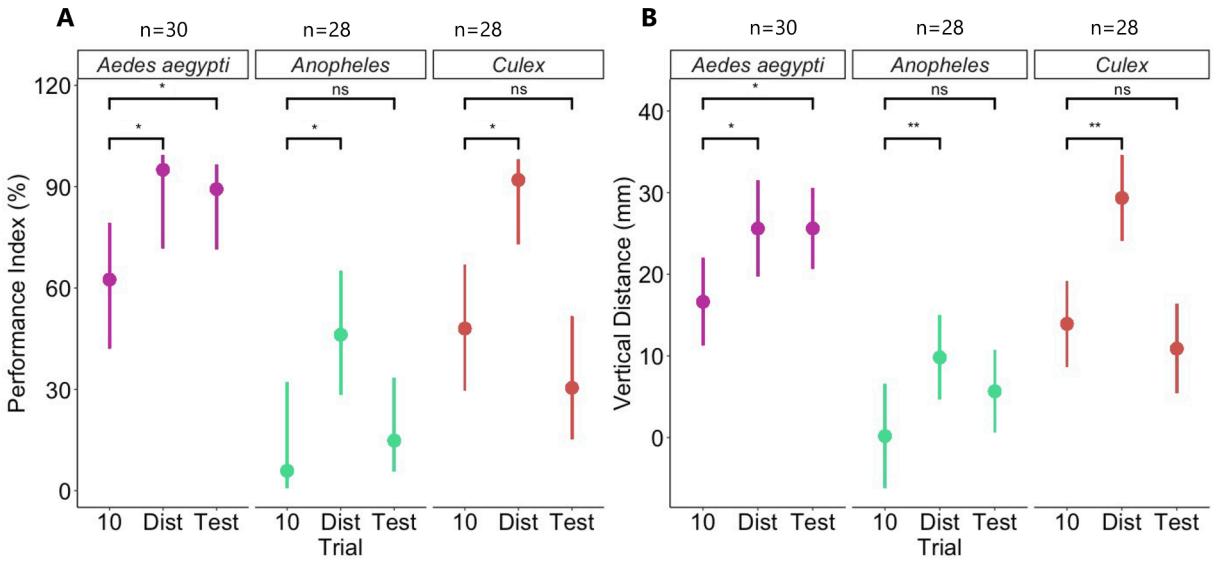

Fig. 6. Dishabituation. A) Performance Index of individuals responding to the visual stimulus in the 10th trial, the *disturbance* phase and in the *test* phase). B) Vertical distance travelled by individuals responding to the visual stimulus in the 10th trial, n the disturbance phase and in the *test* phase. Dist = disturbance, i.e. the mechanical stimulation between the two trials. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

induced by training (Fig. 6). *Culex* and *Anopheles* remained not responsive even after the mechanical disturbance. Yet, all three species evinced an increase in responsiveness when the mechanical disturbance was applied (*Ae. aegypti* PI: 95% CI [0.26, 4.61], P = 0.028; VD: $t_{67} = 2.42$, P = 0.018; *Culex* PI: 95% CI [0.81, 4.27], P = 0.004; VD: $t_{68} = 3.72$, P < 0.001; *Anopheles:* PI: 95% CI [0.46, 4.78], P = 0.018; VD: $t_{65} = 2.72$, P = 0.008; Fig. 6). *Ae. aegypti* showed a significant difference between the 10th trial and the *test* phase for both Performance Index and vertical distance (PI: 95% CI [0.15, 3.06], P = 0.030; VD: $t_{67} = 2.64$, P = 0.010). In contrast, the PI and VD of *Culex* and *Anopheles* were not significantly different between the 10th trial and the *test* phase (*Culex* PI: 95% CI [0.26, 4.61], P = 0.028; VD: $t_{68} = -0.75$, P = 0.456; *Anopheles:* PI: 95% CI [-1.96, 0.45], P = 0.221; VD: $t_{65} = 1.49$, P = 0.141; Fig. 6).

5. Discussion

The goal of the present work was to introduce a novel automated system for evaluating the diving response of mosquito larvae, to validate it with insects belonging to different species and having different origins. We showed that the tracking method and the algorithms developed revealed as useful, rendering accurate sets of data and assuring replicability. Automated tracking methods facilitate behavioural quantitative analyses (e.g. Panadeiro et al., 2021). In our work, different behavioural variables could be quantitatively analysed, allowing comparing performances across mosquito species.

We have been able to investigate habituation in mosquito larvae. As expected (Baglan et al., 2017), *Ae. aegypti* larvae were able to habituate to a visual stimulus initially perceived as dangerous, and control groups allowed to distinguish habituation from fatigue and sensory adaptation (Thompson, 2009). For *Culex* and *Anopheles,* a significant decrease in the escape response occurred and convergent evidence supported the occurance of habituation in these mosquitoes also. On the one hand, the nature of the stimulus (a passing shadow) and the time elapsed between the last training essay and the test (i.e. several minutes), make sensory adaptation unlikely. On the other hand, the intense response triggered by mechanical disturbance allows excluding motor fatigue.

We calculated two main variables, the Performance Index (PI) and the Vertical Distance (VD) travelled by the larvae. PI was conceived as an easy-to-use binary variable to determine the proportion of individual response to the visual stimulus. This variable is analogous to observations that would have been made by a human experimenter, the major difference was the classification process. By setting a threshold to classify individuals as moving or not on the basis of their relative movement, we avoided classifying brief spontaneous movements or erratic behaviour as positive responses to the visual stimulus. We also ensured that the response interval was constant over the training (i.e. similar interval for each trial). This step was crucial especially for very active species such as *Ae. aegypti* (Jackson, 1953; Lutz et al., 2020). A characteristic of the PI is that the threshold was defined in advance, as a minimum intensity of movement for the individual to be considered as responding. In addition, a filter was applied to eliminate 'false zeros' in our zero-inflated model (Zuur et al., 2009), i.e. when individuals could not respond due to their position being at the bottom of the cuvette during the stimulation. Finally, the automated filtering and classification steps provided a robust way to keep constant the selection process over time (i.e. avoiding inter- and intra-observer variability). Thus, quantifying the response of mosquito larvae was based on objective replicable criteria instead of relying on subjective appreciation.

The Vertical Distance (VD) variable was designed to quantify the intensity of the escape response. Upon successive occurrence of the same stimulus, the intensity of a behavioural response may vary or even be completely inhibited (Evans et al., 2019). Here, VD refers to the biological escape response of mosquito larvae, which occurs primarily in the vertical direction, as described by Clements (1999).

Individual displacement was also evaluated in order to quantify spontaneous activity, using the variable Absolute Vertical Distance (AVD), i.e. the total distance travelled during all the ITI periods. Understanding the kinematics of mosquito behaviour using VD or AVD has other advantages. For instance, it allows the interpretation of movement data in a specific context by discriminating between resting period and activity, the direction of displacement, gliding motion, wriggling bouts counts, number of diving events, time spend per area, foraging behaviours, etc (Chandrasegaran et al., 2018; Lutz et al., 2020).

All the three Culicidae studied are part of the neuston (i.e. organisms associated to the water surface, either above or underneath) and, at the same time, they differ in their behaviour. *Ae. aegypti* was the most active during training and the most sensitive to the visual stimulus while *Anopheles* was the least responsive and spontaneously active, and *Culex* was in-between.

Overall, our mosquitoes significantly decreased their response

during the training phase. This variation in their responsiveness to a visual stimulus is the result of a trade-off between avoiding predation, maintaining oxygen levels and conserving energy reserves for adult emergence (Awasthi et al., 2015; Baglan et al 2017; Pientrantuono et al., 2021).

All individuals in *Control No. 1* group (i.e., *disturbance*) strongly responded to the mechanical stimulation. While our visual stimulus simulated a flying predator (Tomsic et al. 2009), the mechanical disturbance could illustrate the sudden movement of waves caused by an aquatic predator (e.g. dragonfly larvae, fish, certain mosquito larvae), and could explain the intense response to vibration of the larvae (Clements, 1999).

Finally, we found a significant difference in dishabituation in larvae, as has been the case in crabs inhabiting different habitats (see review by Tomsic et al., 2009). Yet, the lack of response at the test phase in *Control No. 1* raises the question on potential differences in learning and memory abilities across species.

In summary, we present here an automated tracking system, which revealed to be reliable, accurate and time-saving, for investigating habituation in mosquito larvae. This learning paradigm proved to be an adequate approach for studying a variety of biological questions related to mosquito cognitive abilities (Baglan et al., 2017, Pientrantuono et al., 2021, this paper) as well as the neurological impact of pollutants (Baglan et al., 2018). Other questions which could be addressed using a similar approach range from basic neurobiological mechanisms underlying, for instance memory consolidation and persistence, to ecological problems, as the impact of environmental conditions on cognition.

CRediT authorship contribution statement

Martin Dessart: Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Miguel Piñeirúa:** Data curation, Formal analysis, Methodology, Resources, Software, Software, Validation, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104535.

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