Habituation leads to short but not long term memory formation in mosquito larvae

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ABSTRACT

In animals, memory allows to remember important locations and conserve energy by not responding to irrelevant stimuli. However, memory formation and maintenance are metabolically costly, making it worthwhile to understand the mechanisms underlying different types of memory and their adaptive value. In this study, we investigated the memory persistence of *Aedes aegypti* mosquito larvae, after habituation to a visual stimulus. We used an automated tracking system for quantifying the response of mosquito larvae to the passage of a shadow, simulating an approaching predator. First, we compared different retention times, from 4 min to 24 h, and found that mosquito larvae only exhibited memory capabilities less than 3 h after training. Secondly, we investigated the role of inter-trial intervals in memory formation. In contrast to other aquatic invertebrates, mosquito larvae showed no long-term memory even at long inter-trial intervals (i.e., 5 min and 10 min). Our results are discussed in relation to the ecological constraints.

1. Introduction

To retain information, or memory, is a crucially adaptive cognitive ability in animals (Menzel, 1999). The adaptive value of memory is related to the ability to make quick and accurate decisions when faced with a situation similar to one previously experienced (Menzel and Benjamin, 2013). Memory allows animals to avoid harmful situations, to remember important locations or specific information, and to avoid energy loss by not responding to irrelevant stimuli; in other words, memory contributes to overall fitness (Couto et al., 2023). At the same time, memory formation and maintenance have different costs (Niven and Laughlin, 2008). As the brain is metabolically expensive, the resources allocated to encode, consolidate, and access information generate important expenditures (Kandel, 2001). Different types of memory coexist, defined by their duration and the physiological processes involved in their development. They end up being adaptive or not depending on the context. For instance, in stable environments, where the probability of encountering a certain situation again is high, it may be adaptive to invest in long-term memory. In a rapidly changing environment, however, it may be better to prioritise short-term memory (Pull et al., 2022).

The properties and the physiological mechanisms underlying the different types of memory have been studied in many invertebrate models, notably in the fruit fly *Drosophila melanogaster* (Tully et al., 1994) and the honey bee *Apis mellifera* (Menzel 2001a). In addition, habituation to visual stimuli and memory has been well characterised in the mudflat crab *Neohelice granulata* (Tomsic and Silva, 2023). These experiments provided insights about the ecological relevance of memory duration according to the habitat. In a study by Tomsic et al. (1993), the authors compared the habituation of two related semi-terrestrial crabs that occupy different habitats, *Neohelice granulata* and *Pachygrapsus marmoratus*. By analysing the influence of diverse parameters on visual habituation performances (e.g., individual size, number of trials), the authors showed that habituation is species-dependent and that contextual cues are memorised differently. Tomsic et al. (1993) concluded that ecology played a major role in the origin of these differences. Indeed, *Neohelice granulata* crabs live in self-dug burrows, closed to the mud substrate and surrounded by conspecifics and halophyte vegetation. On the other hand, *Pachygrapsus marmoratus* live on rocky outcrops, close to the sea and without vegetation. So, a shadow passing over *Neohelice* crabs would induces stronger and longer habituation because it represents an ambiguous signal (e.g., grass undulation), whereas for *Pachygrapsus* crabs, the probability of being an actual flying predator would be higher in their environment which is poor in objects passing overhead (Tomsic et al., 1993), resulting in a weak habituation response in the latter.

A key parameter for habituation and the mesic mark it can generate, is the inter-trial interval (Giurfa et al., 2009). Short inter-trial intervals (e.g., from few seconds to few minutes) are more likely to reinforce short-term memory, which relies on neural facilitation (i.e., increase in synaptic strength) and reversible changes (Hemmi and Tomsic, 2012), but not long retention. In contrast, long inter-trial intervals will lead to

the formation of long-term memory, which depends on the activation of specific genes leading to new protein synthesis and structural changes in neural circuits (Tomsic et al., 1996; reviewed in Margulies et al. 2005 in Drosophila). In between, intermediate inter-trial intervals produce intermediate memory, which involves synaptic consolidation through the activation of specific kinases (e.g., cAMP-dependent protein kinase PKA) and early gene expression (Tomsic and Romano, 2013). While the duration of inter-trial intervals has been empirically tested, these types of memory have also been described in several taxa (Izquierdo et al., 1998; Menzel 2001b; Tully et al., 1994).

In this work, we investigated the ability to develop memory after learning in an aquatic insect, the mosquito larva (*Aedes aegypti*). Mosquito larvae spend most of their time hanging from the water surface. When a stimulus is perceived as a potential danger, larvae dive (Clements, 1999). If the stimulus turns out to be innocuous upon repeated occurrences, larvae no longer respond to further stimulation due to habituation, a form of non-associative learning, potentially forming a mnesic trace (Baglan et al., 2017; Dessart et al., 2023).

Although much attention has been paid to cognition in adult mosquitoes, this is the first study to investigate the memory of mosquito larvae. In freshwater ecosystems, mosquito larvae are part of the neuston (i.e., organisms living at the water surface). They are therefore surrounded by unpredictable aquatic and aerial predators such as dragonfly larvae or water striders (for review see: Vinogradov et al., 2022). In this type of environment, a shadow repeatedly casting over the water surface in a short period of time is likely to be projected by the same object, whereas a shadow projected over the water hours later could be produced by a different moving body. In this situation, we could expect that mosquito larvae stop to respond to the repetition of an aversive stimulation in the short term, while resetting their responsiveness in the long term, i.e., not to remember, would be a more adaptive strategy.

In addition to very-well studied aquatic invertebrates such as the sea hare *Aplysia californica* (Glanzman, 2009) or the crab *Neohelice granulata* (Tomsic et al., 2017) which exhibit remarkable forms of long-term memory, other freshwater organisms also showed consistent long-term memories, as for example crayfish *Procambarus cubensis* up to 24 h (Abramson et al., 2005), great pond snails *Lymnaea stagnalis* up to 3 days (Lukowiak et al., 2003), and water fleas *Daphnia sp.* up to 6 days (Ringelberg and Gool, 1995). Since long-term memory has been demonstrated in several aquatic species, the possibility of long-term memory in mosquito larvae cannot be ruled out without experimental evidence.

On the one hand, the highly unpredictable environment could prioritise the formation of a short-term memory in mosquito larvae. On the other hand, other organisms from similar environments show robust long-term memory. To distinguish between these two hypotheses, we conducted a series of experiments with *A. aegypti* mosquito larvae to investigate (1) how long mosquito larvae could retain information after habituation, and (2) whether the duration of inter-trial intervals would play any role in memory formation.

2. Material and methods

2.1. Animals

A. aegypti eggs (Bora strain) were provided by the INFRAVEC2 project of MIVEGEC-IRD (Montpellier, France). The eggs were reared in a climate-controlled room at 25 °C ± 2 °C and under 12 h:12 h light: dark illumination regime (lights on at 8:00). The larvae were maintained in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). Fourth-instar larvae were used in all the experiments to maximise the chances of encountering robust cognitive abilities. In addition, larger larvae allow a more precise tracking. All animals were reared and manipulated according to ethics regulations applied in the European Union.

2.2. Experimental apparatus

The experimental apparatus 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 x 30 cm), located above the cuvettes (Fig. 1A). 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 (for more details, see Dessart et al., 2023). The projected shadow induced naive larvae to dive vertically, escaping from potential danger. After repeated presentations of the shadow, the escape response decreased due to habituation, a form of non-associative learning (Dessart et al., 2023).

2.3. Experimental procedure

The experimental procedure included a training phase and a test phase. During the training phase, individuals were presented with a shadow 10 consecutive times (trials), separated by a specific inter-trial interval (ITI). The stimulus was a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board. During a trial, the stick turned 100∘ and returned back to the resting position, during 3 s. After the 10th shadow presentation, the larvae rested for a specific period (retention time) before a final presentation of the shadow (test phase).

Two experiments were set up to evaluate the duration of memory and possible effects of ITI on the duration of memory. First, 6 treatments of 20——30 individuals were established, each trained with 2-min ITI and tested at different times after the training, ranging from 4 min to 24 h (i. e., Treatment 1 = 4 min, Treatment 2 = 30 min, Treatment 3 = 1 h, Treatment 4 = 2 h, Treatment 5 = 3 h, Treatment 6 = 24 h, Table 1). These intervals were chosen to investigate the memory persistence of mosquito larvae. The order of the treatments was pseudorandomised. Then, a new set of larvae were trained with 2 min ITI, 5 min ITI or 10 min ITI, and retention tested at 24-hour (i.e., Treatment 6 = 2 min ITI, Treatment 7 = 5 min ITI, Treatment 8 = 10 min ITI, Table 1). The order of the treatments was also pseudorandomised, and six replicates of 10 individuals were trained per day, resulting in 6 consecutive days of training (Table 1).

Training and testing took place in the afternoon, from 12 h to 19 h. Individuals were removed from the database if they transformed into pupae during the training or retention period (n = 1), if they remained motionless throughout the entire training period (n = 1) or if the tracking failed to distinguish the individual (n = 2) (Table 1). A total of 205 individuals from 21 replicates were kept for the analysis.

2.4. Data analysis

Each replicate was video recorded, and the individual trajectory was extracted using a tracking algorithm previously used by Dessart et al. (2023) (Fig. 1B). We first applied a detection rate by comparing the number of frames successfully identified by the tracking algorithm with the theoretical maximum number of frames. All videos were analysed with a minimum detection rate of 82.5 % (Table 1). Two analyses were then performed on individual trajectory.

To assess learning and memory abilities, we considered the stimulus response corresponding to the behavioural response of individuals during the 3-seconds trial period of the shadow passage (Fig. 1C). Using these data, we first excluded individuals that were at the bottom of the cuvette at the start of a trial (below 9/10th of the cuvette length, 26.1 % of trials removed, Table 2). We then calculated the variable Vertical
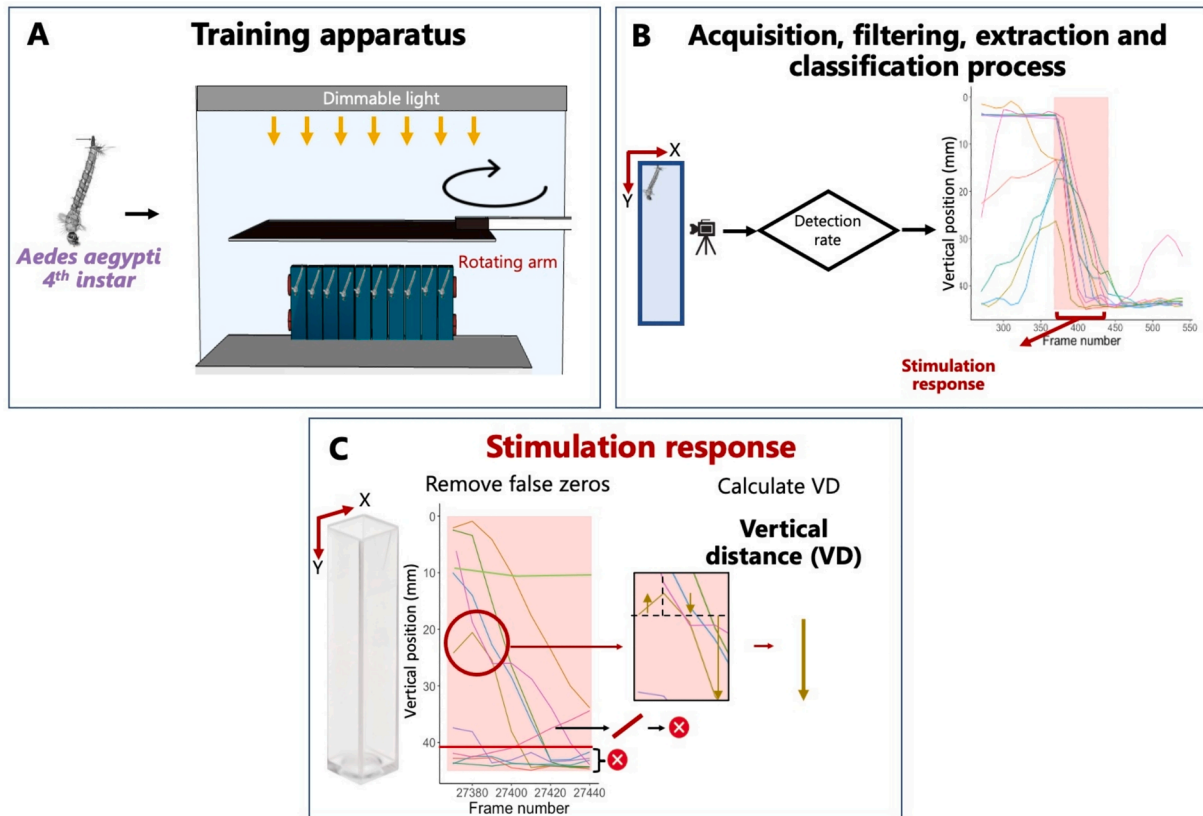
Fig. 1. Schematic of the experimental protocol. A) *Aedes aegypti* larvae were reared in the laboratory and trained on the apparatus at the fourth larval. B) Experiments were video-taped and individual trajectories were extracted. C) We analysed the behavioural response during the aversive stimulus, using the metric vertical distance (VD). This variable was quantitative and calculated as the relative sum of the distance travelled vertically towards the bottom of the cuvette. In addition, two filters were applied to exclude individuals that were at the bottom of the cuvette during the first few frames of the stimulus and individuals that moved upwards during the 3-second stimulus period.

Table 1
Details of the two experiments conducted. Each replicate represents 10 individuals (or less where indicated) trained during one session.

Replicate	Group	Retention	ITI	ID number	Detection rate	Vertical length (px)	Comment
1	1	4 min	2	10	0.82	399.4	
2	1	4 min	2	10	0.83	403.8	
3	1	4 min	2	10	0.95	401.6	
4	2	30 min	2	10	0.93	403.3	
5	2	30 min	2	10	0.92	400.5	
6	2	30 min	2	10	0.96	400.7	
7	3	1 h	2	10	0.97	400.6	
8	3	1 h	2	10	0.89	404.3	
9	3	1 h	2	9	0.93	401.1	ID#10 not tracked
10	4	2 h	2	9	0.98	406.6	ID#6 never moved at all
11	4	2 h	2	10	0.97	400.8	
12	4	2 h	2	10	0.92	405.6	
13	5	3 h	2	8	0.97	400.8	ID#7 transformed in pupae
14	5	3 h	2	10	0.9	399.7	
15	5	3 h	2	10	0.98	404	
16	6	24 h	2	10	0.98	401.9	
17	6	24 h	2	10	0.95	403.4	
18	7	24 h	5	9	0.85	403.6	ID#8 not tracked
19	7	24 h	5	10	0.99	400.8	
20	8	24 h	10	10	0.9	400	
21	8	24 h	10	10	0.92	401.8	

The retention column refers to the time between training and test. ITI = inter-trial interval used during the session. ID number corresponds to the number of individuals for each replicate. Detection rate was calculated as the ratio between the maximum number of frames and the actual number of frames identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured by the tracking software on each video.

Table 2
Summary of the filtering steps. Between 19 and 30 individuals were trained for each species.

2 MIN ITI						5 MIN ITI	10 MIN ITI	All
4 MIN	30 MIN	1H	2H	3H	24H	24H	24H	Total
30	30	29	29	28	20	19	20	205
320	330	319	319	308	220	199	220	2235
236	253	212	256	236	159	147	152	1651
26.3 %	23.3 %	33.5 %	19.7 %	23.4 %	27.7 %	26.1 %	30.9 %	**26.1 %**
230	250	205	255	235	158	140	149	1622
2.5 %	1.2 %	3.3 %	0.4 %	0.4 %	0.6 %	4.8 %	2.0 %	1.8 %
28.1 %	24.2 %	35.7 %	20.1 %	23.7 %	28.2 %	29.6 %	32.3 %	**27.4 %**

If the individual's position was near the bottom at the beginning of a trial, the response to that trial was removed, accounting for a total of 26.1% of removed trials. Individuals that moved upwards during a trial were also removed for that particular trial, accounting for 1.8% of trials.

Distance (VD) as the vertical downward distance travelled by each individual during the 3 s stimulus onset (Dessart et al., 2023). Using VD, we excluded individuals that travelled to the top of the cuvette during a trial (i.e., that travelled more than 10 mm upwards, 1.8 % of trials removed, Table 2). A total number of 205 individuals and 1622 trials were retained for the analysis (Table 2).

3. Statistical analyses

3.1. Data sharing

All the results were analysed using R version 4.1.1 (2021–08- 10) (https://cran.r-project.org/). The data and the R programs used in this study are available at: https://github.com/martindessart/Brain _Like_A_Sieve.

3.2. Data filtering

For all treatments, we verified that number of trials deleted by the criterion did not depend on the trial number (similar as Dessart et al., 2023). Briefly, we applied a Chi-square goodness of fit test to verify that the larval position did not change (i.e., increase or decrease) across trials.

3.3. Learning and memory performance

We first modelled learning performance using Generalised Additive Model (GAM) to provide a visual estimate of the training period. We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE in the *mgcv* package (Wood, 2017). We then evaluated the learning performance of each treatment by comparing the response in the 1st trial to the response in the 10th trial and in the Test phase. For each treatment, we used a linear mixed effects model, choosing VD as the response variable, trial as fixed factor and individual identity as random factor. We checked the homogeneity of the distribution of variances and residuals using the *DHARMa* package (Hartig, 2022). We evaluated the pairwise comparisons using the *emmeans* package with Tukey correction for 3 estimates (Lenth, 2021).

3.4. Test comparison

To compare the duration of memory across treatments, we compared

the response in the Test phase by using a linear mixed effects model with VD as the response variable, the retention time as fixed factor and individual identity as random factor. We followed the same procedure as before to assess pairwise comparisons.

3.5. Learning efficiency

We also estimated how quickly larvae would significantly decrease their response as a function of the ITI, to estimate how many trials would be sufficient to induce a significant decrease in response. To answer, we compared the response at the 1st and the 2nd trial, using a linear mixed effects model as described above.

4. Results

4.1. Data filtering

To analyse the global position of the larvae over time, we compared the proportion of deleted trials by the two criteria between trials (similarly as Dessart et al., 2023). For each treatment, the deleted trials were not specific to a trial number: Treatment 1: $\chi^2 = 7.53$, df $= 10$, P $= 0.674$; Treatment 2: $\chi^2 = 3.6$, df $= 10$, P $= 0.964$; Treatment 3: $\chi^2 = 3.91$, df $= 10$, P $= 0.951$; Treatment 4: $\chi^2 = 6.81$, df $= 10$, P $= 0.743$; Treatment 5: $\chi^2 = 6.41$, df $= 10$, P $= 0.780$; Treatment 6: $\chi^2 = 9.68$, df $= 10$, P $= 0.469$; Treatment 7: $\chi^2 = 15.02$, df $= 10$, P $= 0.131$; Treatment 8: $\chi^2 = 6.93$, df $= 10$, P $= 0.732$.

4.2. Learning performance

For all treatments, the behavioural response of the larvae decreased with successive stimulations (Fig. 2, Fig. 4A). To model this behavioural response, the P-spline from GAM models was the best smoothing function, similar to as previous work (Dessart et al., 2023). For all treatments except Treatment 7, the Vertical distance VD was higher in the 1st than in the 10th trial: Treatment 1: t-ratio $= 3.943$, df $= 45$, P < 0.001; Treatment 2: t-ratio $= 2.590$, df $= 46$, P $= 0.03$; Treatment 3: t-ratio $= 2.626$, df $= 37$, P $= 0.03$; Treatment 4: t-ratio $= 2.704$, df $= 49$, P $= 0.03$; Treatment 5: t-ratio $= 2.540$, df $= 46$, P $= 0.04$; Treatment 6: t-ratio $= 2.957$, df $= 30$, P $= 0.02$; Treatment 8: t-ratio $= 3.315$, df $= 28$, P < 0.01 (Fig. 3A, Fig. 4B). For Treatment 7 (i.e., ITI $= 5$ min and retention time
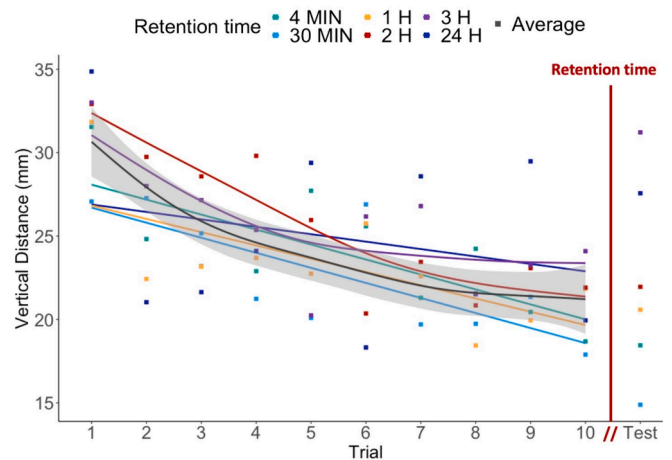

Fig. 2. Habituation curves for larvae trained with 2 min inter-trial interval and with several retention times. 4 min (green), 30 min (cyan), 1 h (yellow), 2 h (red), 3 h (purple), 24 h (dark blue) retention time. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95 % confidence interval for the average response. Points indicate mean values.
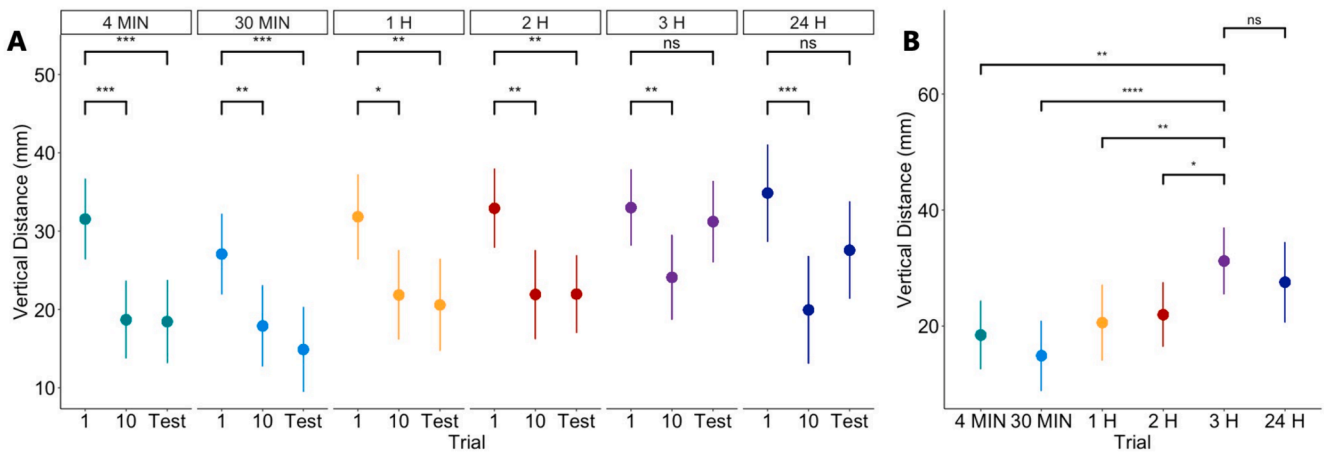
Fig. 3. Mosquito larvae retain visual information up to 2 h after habituation. A). For each retention time, vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the test trial. B) Comparison of the vertical distance travelled by individuals during the test trial. 4 min (green), 30 min (cyan), 1 h (yellow), 2 h (red), 3 h (purple), 24 h (dark blue). Points indicate mean values and bars indicate +- 95 % confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
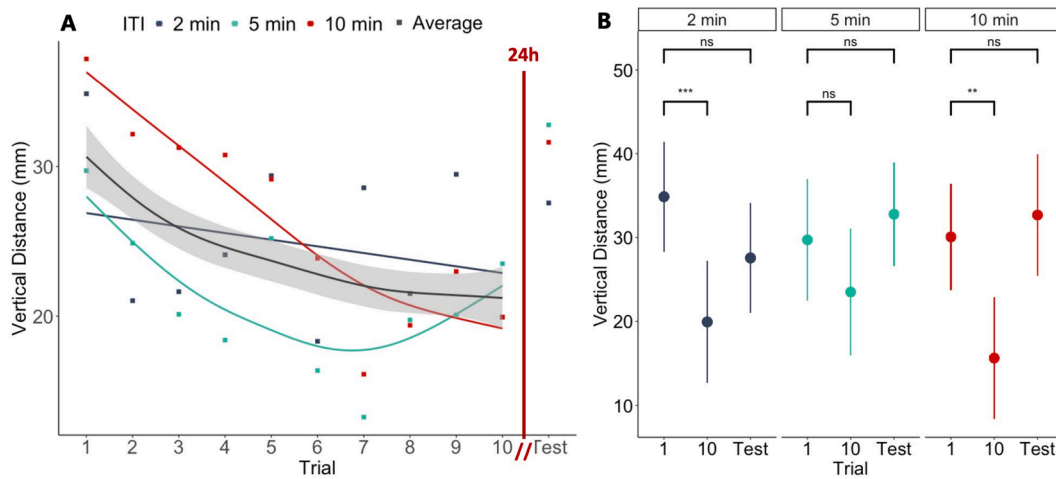

Fig. 4. Learning and memory performance of individuals is independent of the inter-trial interval. A) Habituation curves for individual training with 2 min (dark grey), 5 min (green), 10 min (dark red) inter-trial intervals. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time of 24 h. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95 % confidence interval for the average response. B) For each inter-trial interval, the vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the Test trial. Points indicate mean values and bars indicate +- 95 % confidence intervals. NS, not significant; **P < 0.01, ***P < 0.001.

= 24 h), although the response decreased after the 1st trial, the difference between the 1st and the 10th trial was not significant: t-ratio = 1.088, df = 32, P = 0.53 (Fig. 4B). This difference may be due to the small number of trials retained for this comparison (1st trial: n = 13; 10th trial: n = 12).

4.3. Effect of the retention time on memory performance

To investigate how long the information from the training would be stored in the mosquito larval brain, we compared the response during the Test phase for different retention times. First, we compared the response at the 1st trial to the Test phase (Fig. 3A). For retention times up to 2 h, the response in the Test phase was lower than in the 1st trial: Treatment 1: t-ratio = 3.823, df = 46, P < 0.01; Treatment 2: t-ratio = 3.354, df = 46, P < 0.01; Treatment 3: t-ratio = 2.529, df = 37, P = 0.04; Treatment 4: t-ratio = 2.906, df = 45, P = 0.02; Treatment 5: t-ratio = 0.520, df = 46, P = 0.86; Treatment 6: t-ratio = 1.533, df = 29, P = 0.30 (Fig. 3A). We also compared the response at the Test phase between the highest response (Treatment 5 = 3 h) with other Treatments (Fig. 3B).

The response at the Treatment 5 was higher than Treatment 1: t-ratio = 3.097, df = 100, P < 0.01, Treatment 2: t-ratio = 3.874, df = 101, P < 0.001, Treatment 3: t-ratio = 2.366, df = 103, P = 0.02 and Treatment 4: t-ratio = 2.319, df = 98, P = 0.02. The response at the Treatment 6 was no different from Treatment 5: t-ratio = 0.704, df = 105, P = 0.48, Fig. 3B).

4.4. Effect of intertrial interval on long-term memory

As the larvae showed no retention after 3 h, we investigated whether 2 min ITI was sufficient to induce a long-term memory. We trained larvae using the same procedure, but with an increased ITI of 5 min and 10 min. We compared the response in the Test phase after 24 h of retention time for 3 ITI: 2 min, 5 min and 10 min. The difference between the 1st trial and the Test phase was not significant for any of the three Treatments: 2 min ITI: t-ratio = 1.533, df = 29, P = 0.29; 5 min ITI: t-ratio = -0.596, df = 28, P = 0.82; 10 min ITI: t-ratio = -0.550, df = 25, P = 0.85 (Fig. 4B).

4.5. Effect of intertrial interval on learning efficiency

To characterise the speed of learning, we compared the response between the 1st and the 2nd trials for the 3 ITIs. The difference between the 1st and the 2nd trials was only significant for the 2-min ITI: t-ratio = 2.955, df = 15, P < 0.01; 5 min ITI: t-ratio = 0.836, df = 13, P = 0.42; 10 min ITI: t-ratio = -0.069, df = 16, P = 0.94 (Fig. S1).

5. Discussion

In this work, we took advantage of an automated system to investigate memory persistence in the 4th instar of *A. aegypti* mosquito larvae. Two distinct questions were addressed: how long mosquito larvae could retain information following a habituation learning paradigm, and whether habituation training with longer inter-trial intervals would induce long-term memory in mosquito larvae or not. In the first part of this study, we found that following a habituation protocol, mosquito larvae could retain the information for up to 2 h after 10 trials with 2 min ITI. Yet, no memory was found after 3 h and 24 h of retention. In the second part of this work, we focused on the effect of ITI on the memory persistence at 24 h after training. Equally, we found no long-term memory at 2 min ITI, 5 min ITI and 10 min ITI.

These results support the hypothesis that the ecological context of mosquito larvae would favour short-term memory (Juliano, 2009). First, mosquito larvae live in a wide range of unpredictable environments, where visually hunting predators could be abundant, the structure of the habitat complex and water characteristics (e.g., temperature, light intensity, turbidity) are constantly changing (Crespo, 2011; Yee, 2016; Chandrasegaran et al., 2018). Similar to the crab *Pachygrapsus marmoratus*, which maintains a high response level to a potential flying predator, mosquito larvae could remain safe by quickly forgetting a previous habituation to a moving object, if this reveals to be innocuous (Tomsic et al., 1993). Similarly to *Neohelice granulata* crabs, previous work by our team has shown that habituation learning in *A. aegypti* larvae is context specific (Pientrantuono et al., 2021). However, here the larvae are not tied to a specific location in their aquatic environment and should not be able to learn differentially to less relevant stimuli over a long period of time.

Second, previous studies have shown that mosquito larvae can perceive a wide range of stimuli, such as light contrast, (Liu et al., 2022), conspecific alarm cues (Ferrari et al., 2008), predator vibrations (Roberts et al., 2019), and predator chemical cues (Chandrasegaran et al., 2018). As part of the neuston, these perceptual abilities, combined with their high speed and their diving reflex, allowed them to escape danger (Mellanby, 1958).

Third, when confronted with a repeated stimuli in the same modality, mosquitoes quickly adapt their behaviour in response to experience, i.e., they habituate in few trials. (Baglan et al., 2017; Dessart et al., 2023). Consequently, we can speculate that mosquito larvae should forget quickly and respond strongly to new stimuli after a few times. They should not invest in a costly long-term memory but should favour their own development to minimise time spent in water. This speculation seems interesting when considering the extensive studies on the cognitive abilities of adult mosquitoes, which can retain information for up to 72 h after visual conditioning (Chilaka et al., 2012) and for which remembering information about the last blood-meal is a crucial adaptative behaviour (Vinauger et al., 2014). Furthermore, while adults *A. aegypti* have been shown to learn to associate an a chemical cue with a blood-reinforced thermal stimulus (Vinauger et al., 2014), the question of the potential ability of mosquito larvae to perform associative learning remains open.

To distinguish habituation to other declines in behaviour, previous authors proposed ten criterions (Rankin et al., 2009). The first criterion define habituation. The second, the spontaneous recovery of the response if the stimulus is withheld, and the eighth, dishabituation, were verified in our previous study (Dessart et al., 2023). By comparing the response after training to several controls, we could rule out sensory adaptation, fatigue, and contextual effects. Here, by increasing the intertrial interval to 5 min and 10 min, we observed a decrease in individual spontaneous activity during the training. However, the high individual response at the test phase after 24 h allowed us to rule out all these effects. By increasing the ITI, we also observed that individuals trained with a shorter ITI learned faster than individuals trained with a longer ITI, as revealed by a more rapid decrease in response magnitude along trials at 2 min as compared to others (Fig. S1).

This result supports the fourth criterion proposed by Rankin et al. (2009) for describing the behavioural characteristics of habituation, namely that an increase in stimulus frequency leads to an increase in response decrement.

Some other criteria remain to be explored in the habituation of mosquito larvae, for instance the third criterion which states that after repeated sessions of training, the decrement in response becomes more rapid or more pronounced (Rankin et al., 2009). The use of this criterion may explain how larvae can habituate more quickly to new stimuli shortly after a stimulus has previously appeared, and opens up new questions about the habitat and the cognitive abilities of mosquito larvae.

Finally, the standardised strain reared in the laboratory could influence the ability of *A. aegypti* ability to retain information. Previous authors have shown that a standardized strain could affect the learning abilities and individual fitness (Papaj et al., 1987; Nieberding et al., 2018). Our system is suitable for studying field-reared mosquito larvae and even other small aquatic species that show a comparable escape response.

In this work, we have seen that mosquito larvae have remarkable short-term memory after habituation to an aversive stimulus. This system is well suited to address fundamental questions about larvae abilities to learn and remember, to explore the underlying neurobiological mechanisms, and to ecological perspectives, such as the impact of pollutants or the role of the ecological structure in the development of cognitive abilities.

CRediT authorship contribution statement

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

I have shared the link to my data in the material and methods (line 246-247)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2024.104650.

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