Preimaginal conditioning affects oviposition choices in the silkworm moth (*Bombyx mori*)

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Abstract

Nowadays we can find a number of experiments that have showed the importance of learning in several situations related with survival of many animal species. For instance, knowledge acquired in the early stages of life could be crucial on the choice of egg-laying site. In this study we explored the influence of Pavlovian conditioning of silkworm larvae on their oviposition behaviour as adult female moths. For this, the larvae learning have to survive the metamorphosis and be shown in oviposition choice. In acquisition phase, a larvae group experienced an odour (conditioned stimulus) paired with mulberry leaves (unconditioned stimulus), another one experienced the odour and the mulberry leaves in an unpaired way and the last one experienced the odour alone during this phase. The results show that when these larvae became moths, only the first group preferred to lay their eggs near the odour when it was present during the test, so that associations learned during the larval stage seem to influence oviposition behaviour during adulthood.

Key words: Bombyx mori; insect learning; lepidopteran; preimaginal conditioning; Pavolovian conditioning; oviposition behaviour.

The number of studies dedicated to insect learning is on the rise. A simple search on SCOPUS using the terms “learning” and “insect” retrieves 53 items published in 1999 and 213 items published in 2019. Over the last two decades it has become apparent that several insect species are capable of learning with regard to behaviours such as selecting food (e.g., Cunningham, Moore, Zalucki, & West, 2004; Dukas & Bernays, 2000; Riffell et al., 2008), finding a mate (e.g., Anderson et al., 2007; Anderson, Sadek, Larsson, Hansson, & Thöming, 2013) and rearing offspring (e.g., Cunningham, Jallow, Wright, & Zalucki, 1998; Olsson, Anderbrant, & Löfstedt, 2006). As an example of the first case, Cunningham et al. (2004) conducted an experiment to explore whether learning influences innate preferences for two volatiles, phenylacetaldehyde and α-pinene, in the moth *Helicoverpa armigera*. In a preference test before the Pavlovian conditioning, the moths fly toward the two volatiles, showing some preference for phenylacetaldehyde. During conditioning, a group of moths was exposed to one of the volatiles (Conditioned Stimulus, CS) while having a sucrose solution (Unconditioned Stimulus, US). Two control groups were used: in one of them the moths were exposed to the CS alone, and in the other one no stimuli were presented (naive group). During the test, moths had to choose between the volatiles. The results showed that only the moths in experimental group preferred the volatiles previously associated with the sucrose. In the moths natural environment this learning could be very worthy to discriminate those odours that signal more nutritious sources of food.

Oviposition behaviour refers to behaviours conducted to choice the place where the insect will lay their eggs, so that this behaviour could be related to selecting food and rearing offspring. In Lepidopterans and other insect orders, learning related to oviposition behaviour has attracted particular attention, since that choice seems to be crucial to the survival of offspring. Oviposition could be affected by learning acquired during the adult stage (e.g., Gámez & León, 2018; Rojas & Wyatt, 1999; Zhang, Liu, Wang & Zalucki, 2007). A recent study by Gámez and León (2018), on which the experiment presented here was based, exposed an experimental group of female moths to an odour (CS) paired with mulberry leaves (US). One control group experienced either the CS or US on each trial and a second control group only experienced the CS. At the start of the test phase a male and a female moth were placed in the centre of the box. Half of moths were exposed to the US alone and the other half to the CS. After 24 hours the numbers of eggs that had been laid near the stimulus or in the rest of the box were counted. The results showed that when the CS was present in the test box the experimental group moths laid more eggs near the CS than in the rest of the box. In control groups there was no difference between the number of eggs laid in each zone, showing that experimental moths had associated the odour and the mulberry leaves. Thus, in this experiment Gámez and León (2018) demonstrated that silkworm moths show associative learning and that this kind of learning affects the oviposition behaviour. That is, this experiment shows that learning acquired by moths affects moths' behaviour. But we wondered if learning acquired by larvae could affect moths' behaviour too.

There is some evidence showing that oviposition might be influenced by experience in early life (e.g., Anderson, Hilker & Ofqvist, 1995; Anderson et al., 2013; Moreau, Rahme, Benrey & Thiery, 2008; Olsson et al., 2006). In laboratory and field experiments Anderson et al. (2013) explored how female oviposition choice in *Spodoptera littoralis* was related to plant odour background and showed that the moths preferred to lay the eggs on the host plant on which they were reared as larvae. However, none of these studies explored the role of preimaginal associative learning

(learning that takes place before the moth hatches as an adult) in oviposition behaviour; instead they tried to induce an oviposition preference based on diet.

Moreover, Hopkins' host selection principle states that insects often lay their eggs on those plants on which they fed in the larval stage (e.g., Barron, 2001; Hopkins, 1916). Although this effect might be caused by imprinting of the surrounding conditions on the females during emergence - a phenomenon termed chemical legacy (Corbet, 1985) - it might also be at least partially due to learning in different stages.

There is some evidence that despite the chemical legacy phenomenon, some lepidopteran behaviour seems to reflect the persistence of larval associative learning into adulthood (Blackiston, Silva-Casey & Weiss, 2008), perhaps through survival of larval neurons during metamorphosis, which would enable adult moths to remember associations acquired during the larval stage (e.g., see Tully, Cambiazo & Kruse, 1994 for an example in *Drosophila*).

Hence, since there are few studies dedicated specifically to evaluate whether associative learning acquired during the first stages of an insect's life can remain active after metamorphosis and affect its behaviour (e.g., Blackiston et al., 2008), we intend carry out an experiment similar to Gámez and León (20018), but exploring whether associations acquired by a group of silkworm larvae affects their oviposition behaviour as female adult moths and, thus, whether larval learning persists after metamorphosis.

Method

Subjects

The subjects were 185 silkworm larvae (*Bombyx mori*). They were randomly assigned to three groups: Paired (*n*=64), Unpaired (*n*=59) and CS-Only (*n*=62). After hatching, only the female moths were tested. Thus, groups remained as follows: Paired (*n*=17), Unpaired (*n*=16) and CS-Only (*n*=14). The larvae were purchased from a local vendor.

Apparatus

The caterpillars were housed in six cardboard boxes measuring 33 x 18 x 11 cm (two per group). The moths were housed in another eight boxes with the same dimensions, two for male moths and two for each group of female moths. Two mulberry leaves (*Morus alba*) were used as the US. Three drops (approximately 0.15 ml) of fresh cologne (VéGé) were used as the CS.

Transparent plastic boxes measuring 13 x 13 x 8 cm were used for training. The CS was contained in a 3 cm diameter circular plastic container. Six transparent plastic boxes measuring 32 x 16 x 12 cm were used for testing.

Procedure

The subjects remained in cardboard boxes, where they were fed with mulberry leaves three times a day, until they began the conditioning phase. They were randomly assigned to one of the three groups and at the start of conditioning the groups were separated into different boxes. The experiment was conducted in two phases (see Table 1).

INSERT TABLE 1 ABOUT HERE

Conditioning. Once the silkworms began the fourth instar, they received six sessions of conditioning consisting of 5 trials per session. Trials were separated by a 3 minute inter-trial interval (ITI). Silkworms were trained in groups of 3-5, depending on how many subjects reached the fourth instar at a particular time. The silkworms conditioned at the same time belonged to the same group. The first conditioning session was delivered in the evening of day 1. On the next two days the silkworms received a session in the morning (around 9:00 h) and another one in the afternoon (around 21:00 h). The last session took place in the morning of the fourth day. The central column of Table 1 shows the treatment received by each group.

The silkworms in Paired group experienced the CS and the US together for 5 minutes on every trial (6 sessions x 5 trials x 5 minutes = 150 minutes of exposure to both stimuli). During a trial the stimuli were presented in a side of the box: the CS was presented in a small dish and the two mulberry leaves constituting the US were placed next to the CS. The CS and US were not changed between trials in a session. At the start of a trial the silkworms were placed in the centre of the box and the lid was on; there were no restrictions on their behaviour during the five-minute trial period.

The silkworms in the Unpaired group were exposed to either the CS or US on each trial session. Two stimulus sequences were used; half the subjects received the sequence CS-US-US-CS-CS-US and half received the sequence US-CS-CS-US-US-CS. Thus all animals were exposed to the CS during three sessions and to the US during three sessions. Subjects were randomly assigned to one of the two sequences. For this group the trials lasted 10 minutes so that the total exposure time to the CS and US (3 sessions x 5 trials x 10 minutes = 150 minutes) was the same as for the Paired group.

Finally, the CS-only group received the same treatment as Paired group, except that only the CS was present in the conditioning box. The CS was presented in a central position on the floor of the conditioning box, as it was for the Paired group. Between trials the silkworms were removed from the box and spent a 3-min ITI in boxes that were identical to the conditioning boxes, but completely empty. This group only experience the CS during this phase; hence these larvae remained starved since the beginning of the fourth instar to the fifth instar. This stage includes the fourth instar and the fourth moult. As silkworms do not eat during the moults, the subjects have spent about 4-5 days without eating.

After hatching males and females were separated into different boxes. Males remained together in a box until the test. Females were separated into boxes according to group.

Test. This phase began in the afternoon of the day after hatching. At the start of the test an untrained male and a female moth were placed in the centre of the test box, where they were left for 24 hours. After 24 hours they were removed and the number of eggs was counted, differentiating between those laid in the Stimulus zone and in the Other zone (see Figure 1). In the test phase half of the moths in each group were exposed to the US alone and the other half to the CS alone; thus in half the cases stimulus zone contained the US and in the other half it contained the CS. During the test the US or CS was placed in a central position in the stimulus zone.

INSERT FIGURE 1 ABOUT HERE

Statistical Analysis

The dependent variable was the number of eggs laid in each zone in the test box. The mean numbers of eggs laid in each zone by each group were compared using analysis of variance (ANOVA). The rejection criterion was set at $p < .05$, and effect sizes are reported as partial eta-squared (η_p^2). We also calculated 90% confidence intervals for the effect sizes using the software provided by Nelson (2016).

Results

Figure 2 shows the mean number of eggs laid in each zone by each group, organised by test stimulus (CS or US). The black bars represent the mean number of eggs laid on the Stimulus zone and white bars indicate the mean number of eggs laid on the Other zone. It can be seen that when the US was present all groups laid more eggs in the Stimulus zone; however when the CS was present differences between the groups emerged. The results were analysed using a 3 (Group: Paired vs. Unpaired vs. CS-only) x 2 (Stimulus: CS vs. US) x 2 (Zone: Stimulus zone vs. Other zone) ANOVA. This showed a main effect of Zone, $F_{1,19} = 19.78$, $P < 0.001$, $\eta_p^2 = 0.51$ [CI: 0.21-0.66], and 2 two-way interactions, Group x Zone, $F_{2, 19} = 7.81$, $P < 0.01$, $\eta_p^2 = 0.45$ [CI: 0.12-0.60], and Stimulus x Zone, $F_{1, 19} = 22.74$, $P < 0.001$, $\eta_p^2 = 0.54$ [CI: 0.25-0.69]. There were no other main effects or interactions, $\mathrm{F}_{2, 19} \leq 2.47$, $P \geq 0.111$. The lack of a three-way Group x Stimulus x Zone interaction appears to be due to the similarity of the control groups, as can be seen in the figure.

INSERT FIGURE 2 ABOUT HERE

Planned comparisons were carried out to explore the results of each group. Separate Stimulus x Zone ANOVAs were conducted for each group. In the Paired group there was a main effect of Zone, $F_{1, 7} = 49.20$, $P < 0.001$, $\eta^2_p = 0.87$ [CI: 0.57–0.92] but no interaction, $F = 1.07$, $P = 0.33$. Post hoc analyses showed significant effects of zone in the presence of both the US, $F_{1, 7} = 15.52$, $P < 0.01$, $\eta^2_p = 0.69$ [CI: 0.20–0.81], and CS, $F_{1, 7} = 10.61$, $P = 0.012$, $\eta^2_p = 0.57$ [CI: 0.10–0.76], indicating that the moths laid more eggs on the Stimulus zone than the Other zone when the US was present and, more importantly, when the CS was present.

The same analysis of data from the Unpaired group revealed a Stimulus x Zone interaction, $F_{1, 7} = 29.91$, $P = 0.001$, $\eta_p^2 = 0.81$ [CI: 0.41–0.88]. There was no main effect of Stimulus or Zone, largest $F \leq 0.36$, $P \geq 0.565$. Follow-up analyses exploring the interaction revealed a simple effect of Zone when the US was present, $F_{1, 7} = 21.99$, $P = 0.002$, $\eta_p^2 = 0.76$ [CI: 0.30–0.85], and when CS was present, $F_{1, 7} = 17.71$, $P = 0.004$, $\eta_p^2 = 0.72$ [CI: 0.24–0.83]. These results indicate the moths in the Unpaired group laid more eggs in the Stimulus zone when the US (mulberry leaves) was present but when the CS was present they laid more eggs in the Other zone.

Finally, in the CS-only group there was a Stimulus x Zone interaction, $F_{1, 5} = 9.47$, $P = 0.028$, $\eta_p^2 = 0.65$ [CI: 0.06–0.80], but no main effects of Stimulus or Zone, largest $F \leq 2.97$, $P \geq 0.145$. Follow-up analyses showed that there was a simple effect of Zone when the US was present, $F_{1, 5} = 16.57$, $P = 0.010$, $\eta_p^2 = 0.77$ [CI: 0.20–0.86], but not when the CS was present, $F < 1$. Once again, moths laid more eggs on the Stimulus zone than the Other zone when the US was present, but when the CS was present similar numbers of eggs were laid in each zone.

Discussion

This experiment explored whether Pavlovian conditioning acquired by silkworms during the larval stage would be retained after metamorphosis and affects the adult moths' oviposition behaviour. The results are similar to those reported by Gámez and León (2018).

On one hand, the results showed that in all three groups moths preferred to lay the eggs near leaves of the plant they ate when they were larvae, mulberry, which is consistent with the Hopkins principle (Hopkins, 1916).

On the other hand, the results from the Paired group showed that silkworms exposed to a combination of odour and mulberry preferred to lay their eggs near the odour, if it was present, when they become moths. This indicates that the associations acquired by the larvae can be transferred to the adult moths' oviposition behaviour. In the absence of mulberry leaves, moths from the Paired group preferred to lay their eggs near the odour that had been previously associated with mulberry. This result cannot be explained by moths' exposure to a particular larval environment (some chemical residue of the odour could transfer from the larva to adult as it emerges), because this environment was the same for all subjects. Gámez and León (2018) pointed out that Pavlovian associations acquired in the adult phase are biologically relevant to oviposition behaviour, because oviposition choice influences the foraging of offspring. It seems there is also adaptive value in knowledge acquired by larvae being available to adult moths, as it has been demonstrated in this experiment.

Only in Paired group moths laid more eggs on Stimulus Zone than the Other Zone when the CS was present. As the results from the Unpaired group show a much higher number of eggs on the Other Zone than the Stimulus Zone, we might think that silkworms are capable of acquiring inhibitory associations, i.e. learning to predict the absence of a US from a given CS (Pavlov, 1927). However, retardation and summation tests should be applied to conclude that.

Finally, so that the memories acquired during the larval stage can remain in the adult brain, as we have demonstrated in this study, it is necessary some larval neurons survive the metamorphosis. The metamorphosis of Lepidoptera entails the death of some larval neurons, conservation and remodelling of others and maturation of adult-specific cells (e.g., Truman, 1992). Some of the neurons in the fruit fly's mushroom bodies that are not destroyed in metamorphosis (e.g., Park & Lee, 2018) are involved in olfactory memory (e.g., Fahrbach, 2006; Heisenberg, 1998). Although as far as we know there is no direct evidence about changes suffered by this types of neuron in Lepidopteran metamorphosis, the results of our experiment show that the transition from the larval state to the adult state does not destroy the neurons involved in Lepidopteran olfactory conditioning, unless this kind of learning is possible without this neural structure.

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Table 1. *Experimental design*

Group	**Conditioning**	**Test**
Paired	CS –> US	CS
		US
Unpaired	CS / US	CS
		US
CS-Only	CS	CS
		US

Note. CS: Conditioned stimulus (odor); US: Unconditioned stimulus (mulberry leaves). Arrow indicates simultaneous presentation, whereas the slash indicates uncorrelated presentations.

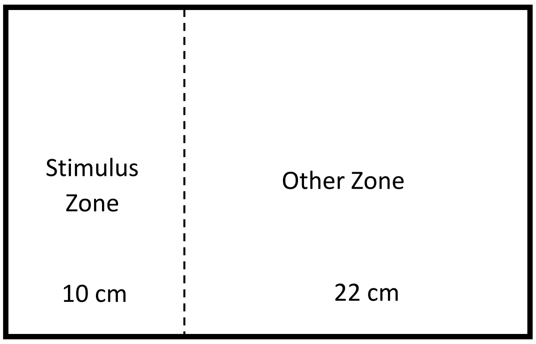
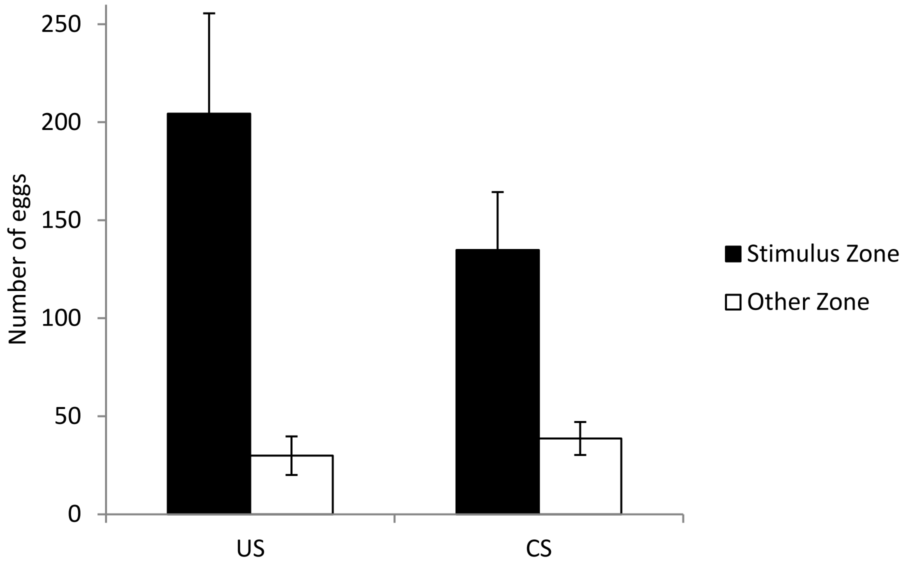
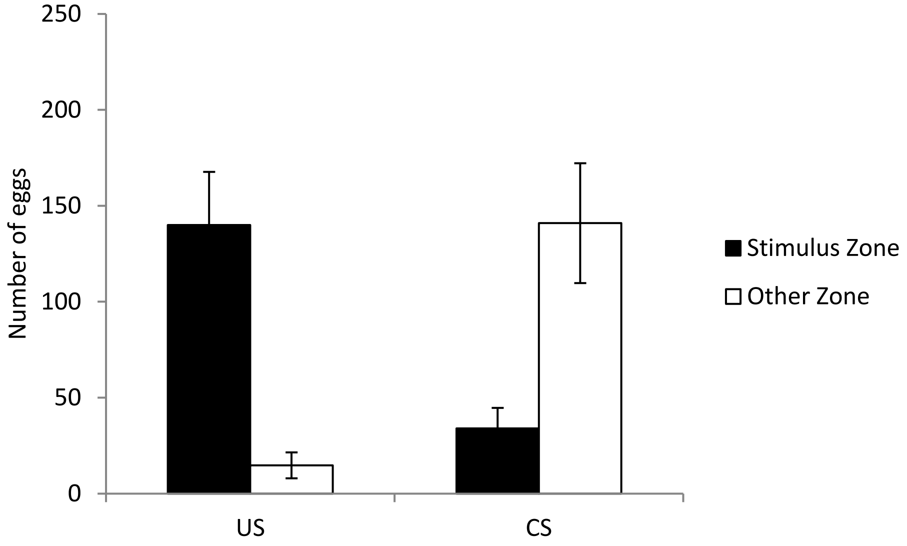


Figure 1. Schematic of the Test box. “Stimulus Zone” is the space in which the stimulus (CS or US) is presented during the test. “Other Zone” refers to the rest of the box.

A


250
200
150
100
50
0
Number of eggs
US
CS
■ Stimulus Zone
□ Other Zone

B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Number of eggs
US
CS
■ Stimulus Zone
□ Other Zone

C

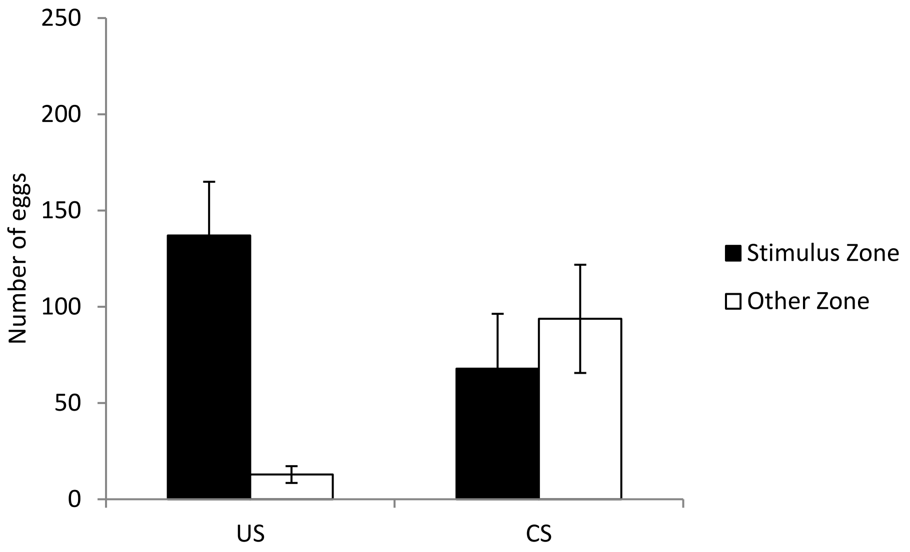

Figure 2. Number of eggs laid during the test in each box zone (Stimulus Zone or Other Zone), depending on whether the stimulus present was the US or the CS. Subjects of the Paired Group are shown in panel A, the Unpaired Group in panel B, and the CS-Only Group in panel C. The left section of each figure shows the results for the moths tested in the presence of the mulberry leaves (US), and the right shows results for the moths tested in the presence of the odor (CS).