SHORT TITLE: LEARNING AND OVIPOSITION

The role of learning in the oviposition behavior of the silkworm moth (*Bombyx mori*)

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Abstract

One of the most relevant behaviors in the life of some insects is oviposition, because reproductive fitness largely depends on the choice of egg-laying site. We report one experiment that explored the influence of Pavlovian conditioning on oviposition choices of the silkworm. Our results show that moths that jointly experienced an odor (conditioned stimulus) and mulberry leaves (the preferred oviposition place for the moths) preferred to lay their eggs near the odor when it was present, whereas moths in which the odor and the mulberry leaves never appeared together showed no preference. This result provides evidence of the important role that a psychological process such as Pavlovian conditioning has for the survival of this species.

Key words: silkworm; lepidopteran; Pavlovian conditioning, oviposition behavior.

Although there are 80 years of study of learning in insects (e.g., Thorpe & Jones, 1937), and such research is growing, research in insect learning is still a minority. Animals must adjust to changing conditions, and they benefit from learning in variable environments with some predictability by using information they have previously acquired. The ability to change behavior after experience is adaptive (e.g., Alcock 1993). The experiment presented here provides new evidence about the learning capabilities of silkworms and the functional advantages obtained by learning in this species.

Scientific evidence has shown the learning capabilities of insects for some crucial behaviors such as searching for food (e.g., Cunningham, Moore, Zalucki & West, 2004; Dukas & Bernays, 2000; Riffell et al, 2008), mate finding (e.g., Anderson et al., 2007; Anderson, Sadek, Larsson, Hansson & Thöming, 2013; see Dukas 2006 for a review) and rearing offspring (e.g., Cunningham, Jallow, Wright & Zalucki, 1998; Olsson, Anderbrant & Lofstedt, 2006). For instance, Cunningham et al. (2004) explored whether learning influences innate preferences for two odors, phenylacetaldehyde and α-pinene, in the nectar-feeding moth *Helicoverpa armigera*. In a preference test, prior to a Pavlovian conditioning procedure, moths flew towards both odors, with a preference for phenylacetaldehyde. During conditioning, a group of moths was exposed to one of the two odors while feeding on a sucrose solution. Two control groups were used for comparison. One group of moths was exposed to odors without allowing feeding while another received no exposure to either the odors or feeding. During the test, moths could choose between the odors. Moths that received the odors paired with feeding showed a clear preference for the odor associated with the sucrose, whereas this change in preference was not seen in the controls. In the moths' natural environment this learning would allow them to discriminate odors that signal more nutritious feeding sources.

This kind of result highlights the relevance of learning for the species' survival. In that theme, other studies have evaluated the adaptive advantage of an associative learning process, such as Pavlovian conditioning, in a variety of taxa such as birds (e.g., Adkins-Regan & MacKillop, 2003; Domjan, Blesbois, & Williams, 1998), fish (Hollis, Dumas, Singh & Fackelman, 1995; Hollis, Pharr, Dumas, Britton & Field, 1997), mammals, (Coria-Avila, 2012; Zamble, Hadad, Mitchell & Cutmore, 1985) and insects (Dukas, 1998; Dukas & Bernays, 2000; Guillette, Hollis & Markarian, 2009).

In an experiment with grasshoppers, Dukas & Bernays (2000) showed the importance of associative learning for optimal growth. These authors arranged two groups of grasshoppers so that the experimental group was exposed to a combination of cues (spatial location, taste, and color) paired with a balanced diet including a suitable proportion of carbohydrates and protein that allows maximal growth, or a deficient diet with no carbohydrates. A control group was unable able to use any of the cues for choosing between the diets. The results showed that grasshoppers in the experimental group were able to use the predictive cues to visit the balanced diet more frequently and ate more of the balanced diet. Hence, the growth rate was higher in experimental than in control group.

Oviposition is one of the most important behaviors for a lepidopteran since choosing the substrate upon which to lay eggs could be momentous for the survival of the offspring. New larvae will be properly fed when they are born surrounded by food without the need to move for foraging. The success of oviposition in the silkworm not only depends on the substrate. There are a number of neural, chemical, physical, environmental, and behavioural factors involved in this behavior. For instance, the number of eggs laid and the time the moth expends ovipositing are influenced by environmental conditions such as temperature, light, humidity, and surface texture. Once the mating is complete and the female moths have found a suitable place, 90% of the eggs are deposited within a 24-hour period after mating. Several researchers have established that higher oviposition rates occur at a temperature of about 25 ±1º C and 80 ± 5% relative humidity (e.g., Singh & Saratchandra, 2004).

There are multiple examples about how larval or oviposition experiences influence later oviposition choices. However, the evidence regarding associative learning in adult lepidopterans in relation to oviposition is scarcer. For instance, several studies state that female moths learn associations between leaf shapes and the chemical compositions required for oviposition (Papaj, 1986), or between the appearance of oviposition substrates and the chemical oviposition stimulants (Traynier, 1984, 1986). Lepidopterans and other insects show an innate preference for a given chemical composition of the substrate. Thus, the plant odor is a valuable cue for this kind of animal to choose their oviposition substrates. For example, Traynier (1986) tested the preference of cabbage butterflies for laying their eggs on discs with different sizes and colors that had been wetted with either a sinigrin solution or water. Sinigrin is a chemical compound in plants like cabbage that releases the oviposition behaviour in lepidopteran species such as the cabbage butterfly. The results showed the individuals preferred to lay the eggs on discs with a similar appearance to those containing the sinigrin solution.

As far as we know, this study and the rest of studies about learning in oviposition behavior explore the insects' ability to associate some features of the plants with its suitability for oviposition. However, the experiment we present in this paper evaluates the silkworm's ability to learn to predict the presence of food through cues different from the food itself. The silkworm only feeds during the larval period. In its adult phase, i.e., when it becomes a moth, it does not feed and survives on the energy reserves created during the larval state. Unlike other lepidopteran species, the silkworm is monophagous, it solely eats mulberry leaves. Therefore, *a priori* the silkworm does not need to learn to choose the most suitable food. Nevertheless, there would still be an adaptive advantage to be able to learn that specific stimuli can predict the presence of mulberry leaves. Such a capacity would allow moths to better locate food sources upon which to lay eggs.

A moth's capacity for Pavlovian conditioning has been clearly demonstrated (see as well Daly & Smith, 2000; Fan, Anderson & Hansson, 1997; Jørgensen, Stranden, Sandoz, Menzel & Mustaparta, 2007). Based on those findings, it can be expected that the preference for oviposition can also be conditioned. Thus, our goal in this experiment was not to explore the influence of previous experience for oviposition, but to specifically test if moths would prefer to oviposit near a stimulus that had been previously associated with the host plant.

The general frame for the experiment presented here is the Hopkins' host-selection principle (HHSP). The principle states that "a species which breeds in two or more hosts will prefer to continue to breed in the host to which it has become adapted" (Hopkins, 1916, p. 353). Related to the HHSP, the natal habitat preference induction hypothesis (NHPI) more specifically states that insect females prefer to lay their eggs on the host species on which they developed as larvae (Davis & Stamps, 2004). Several studies have supported these ideas (e.g., Blackiston, Casey, & Weiss, 2008; Gandolfi, Mattiacci, & Dorn, 2003; Tully, Cambiazo, & Kruse, 1994; but see e.g. Barron, 2001; Janz, Söderlind & Nylin, 2008 for results to the contrary). Hence, it could be expected that the silkworm moths will prefer to oviposit on mulberry leaves, the plant they ate during their larval period. Moreover, if the oviposition of silkworms can be conditioned, when an odor (conditioned stimulus, CS) is paired with mulberry leaves (unconditioned stimulus, US), the moths will lay the eggs near the odor, similarly to way they do on mulberry leaves.

To test those hypotheses, a group of female moths in the “Paired Group” experienced an odor (CS) paired with a mulberry leaf unconditioned stimuli (US). Two control groups were used. One group, “Unpaired Group” experienced the CS and the US unpaired, so that the stimuli were never were presented together and the final “CS-Only Group” experienced only the CS. During the test, one half of each group was exposed to the US and the other half to the CS in a box with a male moth. We counted the number of eggs laid on the area in which the stimulus was presented (the Stimulus Zone) or on the rest of the box (the Other Zone). It was expected that all the moths exposed to the US during the test would lay more eggs on the Stimulus Zone than on the Other Zone, independently of previous conditioning (Davis & Stamps, 2004; Hopkins, 1916). However, during the test with the CS, it was expected that Group Paired would lay more eggs in the area of the box where the CS had been placed (Stimulus Zone). To the contrary, in the presence of the CS, moths in the Unpaired or in the CS-Only groups were not expected to show such spatial preference for laying their eggs. That is, moths were not expected to prefer to lay eggs on the side that contains a stimulus, but only a stimulus that either promotes successful larvae (the mulberry leaves) or one associated with that stimulus as could occur in Group Paired.

Method

Subjects

Subjects were 90 silkworm moths (*Bombyx mori*), 45 males and 45 females. The female moths were randomly assigned to three groups: Paired (N=16), Unpaired (N=16) and CS-Only (N=13). The larvae were purchased from a vendor located through the internet (https://www.milanuncios.com/anuncios/gusanos-de-seda.htm).

Apparatus

For this experiment four cardboard boxes measuring 33 x 18 x 11 cm (*H* x *W* x *D)* were used to house the moths, one of them for male moths and the others 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. For testing three transparent plastic boxes neasuring 32 x 16 x 12 cm were used.

Procedure

In their larval stage the subjects remained in cardboard boxes, where they were fed with mulberry leaves three times a day until they began metamorphosis. After hatching, males and females were separated into different boxes. Males remained together in a box until the test. Females were randomly assigned to one of the three groups and separated into different boxes by group.

The experiment consisted of two phases: conditioning and test.

Conditioning. Female moths received 6 sessions of conditioning with 5 trials each session. After each conditioning trial there was a 3 minute inter-trial interval (ITI). Moths were trained individually or in groups of 2-5 animals, depending on how many moths were ready. The moths conditioned at the same time belonged to the same group. The first session was the first afternoon after hatching. On the next two days moths received a session in the morning (around 9:00 h) and another one in the afternoon (around 21:00 h). Last session was the morning of the fourth day. The central column of Table 1 shows the treatment received by each group during conditioning.

The moths in Paired Group experienced the CS and the US together for 5 minutes on each trial (6 sessions x 5 trials x 5 minutes = 150 minutes of experience with each stimulus). On a trial the stimuli were presented in the middle of the box with the CS presented in the small dish and the US presented by placing two mulberry leaves next to the CS. The CS and US were not changed between trials or between moths in a session. The moths were placed in the center of the box and allowed to freely behave for 5 minutes with the box top covered.

For Unpaired Group, moths in each trial were exposed to CS or US (half of the subjects started with the CS and the other half started with the US). The stimulus sequence used over the six sessions in this group was CS-US-US-CS-CS-US or US-CS-CS-US-US-CS, so that they received 3 sessions with CS and 3 sessions with US. Each subject in this group was randomly assigned to one of the two sequences. Trials in this group lasted 10 minutes so that the exposure time of these subjects to stimuli was the same as in the Paired Group (3 sessions by stimulus x 5 trials x 10 minutes = 150 minutes of experience with each stimulus). Finally, the CS-Only Group received the same treatment as Paired Group, with the exception that only the CS was presented in the conditioning box. The stimuli during conditioning were placed in a central position on the ground of the conditioning box in the same way as in the Paired group. Between trials the moths were removed and spent a 3-min ITI in boxes identical to the conditioning boxes, but these were completely empty.

Test. This phase began the afternoon of the fourth day after hatching and lasted 24 hours. The test started by placing a male and a female moth in the center of the test box. After this time, the moths were removed, and eggs were counted, differentiating those laid on the Stimulus Zone of those laid on the Other Zone (see Figure 1). In this phase half of the moths of each group were exposed only to the US and the other half only to the CS, so that in half of the cases Stimulus Zone represents the area where the US has been presented, whereas in the other half the CS was the stimulus placed on that area. During the test the US or CS were placed in a central position in the Stimulus Zone.

Statistical Analysis

The dependent variable was the percentage of eggs laid in each zone in the test box. This percentage was calculated as indicated by the formula below.

$$\frac{\text{number of eggs in Stimulus Zone}}{\text{number of eggs in Stimulus Zone} + \text{ number of eggs in Other Zone}}\ x\ 100$$

The mean percentage of eggs laid in each zone was compared using analyses of variance (ANOVA). The rejection criterion was set at $p<.05$, and effect sizes were reported using partial eta-squared (η_p^2). Moreover, 90% confidence intervals for the effect sizes were calculated and reported for each analysis using the software provided by Nelson (2016).

Results and Discussion

Figure 2 shows the mean percentage of eggs laid by each group during the test on the Stimulus and Other Zones broken down by the test stimulus (CS or US). Black bars show the mean percentage of eggs laid on the Stimulus Zone and white bars indicate the mean percentage of eggs laid on the Other Zone.

As it can be seen in the figure, all groups laid a higher percentage of eggs in the Stimulus Zone when the US was presented. However, when the CS was presented, differences between groups appeared. To analyze the patterns shown in the graphs a 3 Group (Paired vs. Unpaired vs. CS-Only) x 2 Stimulus (CS vs. US) x 2 Zone (Stimulus

Zone vs. Other Zone) ANOVA was conducted. This analysis found a significant main effect of Zone, $F_{1,39} = 66.14$, $P < .001$, $\eta_p^2 = .62$ [CI: .46- .72], as well as the two-way interactions Group x Zone $F_{2,39} = 7.36$, $P < .01$, $\eta_p^2 = .27$ [CI: .07- .42], and Stimulus x Zone, $F_{1,39} = 21.96$, $P < .001$, $\eta_p^2 = .36$ [CI: .16- .51] and, more interestingly, the Group x Stimulus x Zone three-way interaction, $F_{2,39} = 6.65$, $P < .01$, $\eta_p^2 = .25$ [CI: .06- .40].

To analyze the 3-way interaction we conducted Stimulus x Zone ANOVAs within each group. In the Paired Group, there was an effect of Zone $F_{1,14} = 294.66$, $P < .001$, $\eta^2_p = .95$ [CI: .89- .97] and no interaction, $F < 1$. There were significant, and large, effects of zone both the US stimulus $F_{1,7} = 110.25$, $P < .001$, $\eta^2_p = .94$ [CI: .77- .96], and, of most importance, the CS stimulus $F_{1,7} = 215.54$, $P < .001$, $\eta^2_p = .97$ [CI: .87- .98]

The same analysis focused on the Unpaired Group found a Stimulus x Zone interaction, $F_{1,14} = 16.45$, $P < .001$, $\eta_p^2 = .54$ [CI: .19- .70], although the main effect of Zone approached significance, $F_{1,14} = 4.31$, $P = .057$ $\eta_p^2 = .23$ [CI: .00- .48]. The effect of Zone was significant when the US was present, $F_{1,7} = 94.99$, $P < .001$, $\eta_p^2 = .93$ [CI: .74- .96], but not when CS was present, $F_{1,7} = 1.08$, $P = .332$, $\eta_p^2 = .13$ [CI: .00- .45], indicating the moths in the Unpaired Group laid a similar percentage of eggs on both zones when CS was present during the test.

A similar pattern was observed in the CS-Only Group, where there was a main effect of Zone, $F_{1,11} = 10.23$, $P < .01$, $\eta_p^2 = .48$ [CI: .09- .67], and a Stimulus x Zone interaction, $F_{1,11} = 7.59$, $P = .019$, $\eta_p^2 = .40$ [CI: .05- .62]. The simple effect of Zone was significant when the US was present, $F_{1,5} = 98.36$, $P < .001$, $\eta_p^2 = .95$ [CI: .74- .97], but not with the CS, $F<1$. As in the Unpaired Group, moths laid a higher percentage of eggs on the Stimulus Zone than the Other Zone when the US was present, with no differences between the zones when only the CS was present.

As statistical analyses have confirmed, regardless of whether the moths had been exposed to the uncorrelated CS and US, only to the CS, or to the paired CS-US presentations, in the presence of the US all the moths preferred to lay the eggs near that stimulus (mulberry leaves). This result is consistent with the Hopkins principle (Hopkins, 1916) and the natal habitat preference induction hypothesis (Davis & Stamps, 2004). It is possible that the moths had laid the eggs near the mulberry (the host plant) because it was the plant they ate when they were larvae or simply because it is the only plant present in the box. An additional experiment would be needed to determine the merits of that possibility allowing the moths the possibility of choosing between the host plant and a non-host plant during the oviposition test.

Nevertheless, when the CS was present in the test, the results were different depending on the group. In the Paired Group the percentage of eggs was higher in Stimulus Zone than in Other Zone in presence of the CS. That is, moths that experienced the odor paired with the mulberry leaves preferred to lay their eggs close to the odor when it was present. The oviposition choices were conditioned. When the CS was present, the difference between the number of eggs on the Stimulus and Other zones was not reliable in the Unpaired Group and CS-Only Groups. That the difference appeared only in the group for which the leaves and odor were paired supports that the result found in Paired Group is due to conditioning.

In absence of mulberry leaves, moths prefer to lay their eggs near something, the odor in this case, that had been previously associated with that plant. As we said above, the silkworm does not eat during the adult phase, so the influence of the moth’s food preferences on the oviposition behavior can be precluded. To our knowledge, this is the first clear evidence of learning in an insect species that is economically important as the producer of silk. Moreover, our results support the statement that post-hatching experience can affect oviposition choice (e.g., Olsson et al., 2006), and that one important source of this experience comes from a basic learning process such as Pavlovian conditioning. Thus, silkworms not only are able to use associative learning, but this learning can have an important biological function in this insect.

The behavior studied here is very relevant for a lepidopteran from an ecological point of view. Though the adaptive value of conditioning has been demonstrated in many species (e.g., Domjan et al., 1998; Guillette et al., 2009; Hollis, 1984), it should be noted that studies concerned with Pavlovian conditioning, contrary to the experiment presented here, often show little clear evidence regarding the adaptive value of the conditioned response acquired by the organism, human or otherwise. In the present research, Pavlovian conditioning contributes the foraging of offspring, thus the results reported here evidence that Pavlovian conditioning can provide a clear advantage to silkworms for the species' survival.

Finally, it should be noted that this experiment explored associative learning that has been acquired during the adult phase of the silkworm. Nevertheless, it would stand to reason that classical conditioning will be adaptive if the knowledge acquired by larvae can be used by moths. Such questions can be addressed with new research.

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Table 1. Experimental design

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

Group	**Training**	**Test**
Paired	CS –> US	CS
		US
Unpaired	CS / US	CS
		US

CS-Only	CS	CS
		US

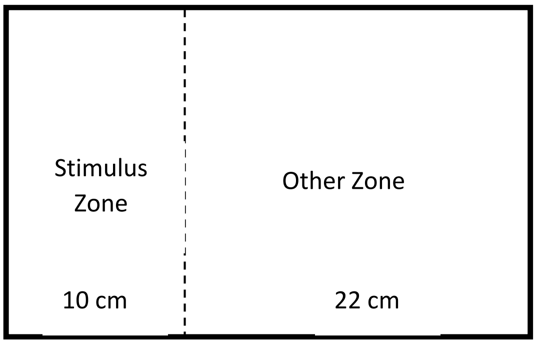
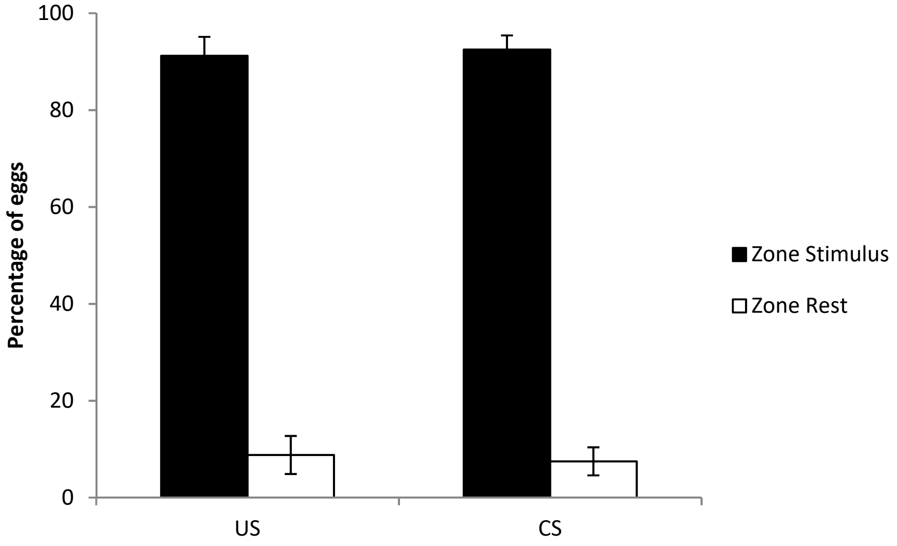
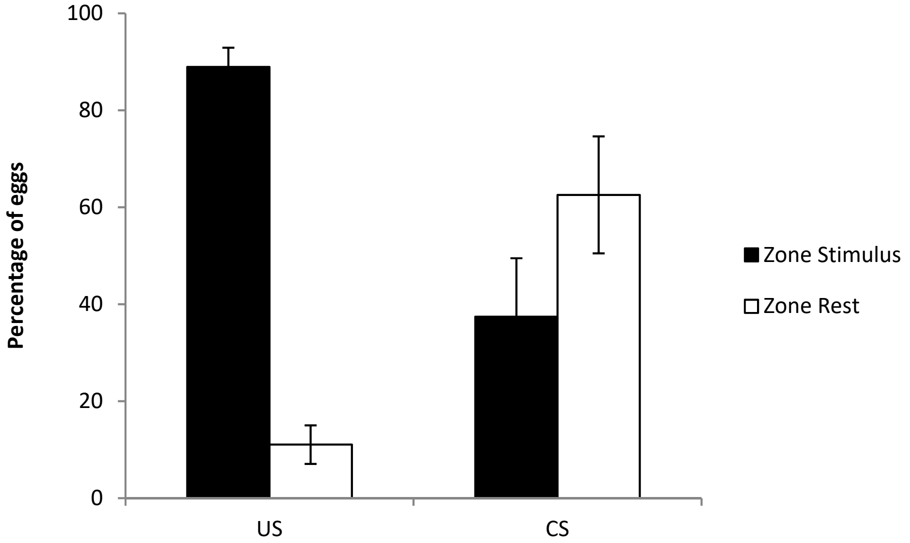


Figure 1. Schematic of the Test box. “Stimulus Zone” is the space in which the stimulus (CS or US) is presented during the test. “Other Zone” refers to the rest of the box.

A


100
80
60
40
20
0
Percentage of eggs
US
CS
■ Zone Stimulus
□ Zone Rest

B



C

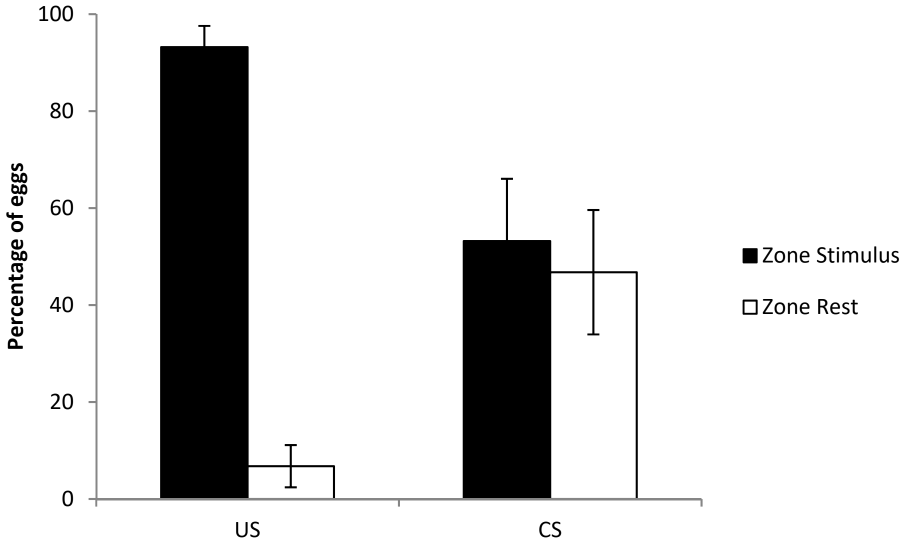


Figure 2. Percentage of eggs laid during the test in each box zone (Stimulus Zone or Other Zone), depending on whether the stimulus present was the US or the CS. Subjects of the Paired Group are shown in panel A, the Unpaired Group in panel B, and the CS-Only Group in panel C). The left section of each graph 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).

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