

Quantifying insect predation in laboratory arenas: the effect of prey acclimation

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Abstract

There are drawbacks associated with current methods used to quantify insect predation. However, there has been relatively little effort to experimentally document the sources of biases associated with different methodologies. In this study, we investigated how methods used experimentally to quantify predation may influence insect antipredator defenses being observed, and thus alter the determination of the number of prey killed by predators, and potentially the conclusions reported in the study. We focused on acclimation period, which represents the time between the point at which a prey individual is made available, for example on a leaf, and when the experiment is initiated. In essence, this is the period of time during which an organism adjusts to the conditions that surround it before a treatment is imposed. We investigated if prey acclimation period on plants influences the antipredator behavior of larval *Orgyia leucostigma* (J.E. Smith) (Lepidoptera: Lymantriidae), and the number of prey killed in the laboratory by the predatory wasp *Polistes fuscatus* (Fabricius) (Hymenoptera: Vespidae). The results of this study provide evidence that acclimation time significantly affects the antipredator behavior observed and mortality of caterpillars exposed to predators. The majority of larvae allowed short acclimation periods did not drop in response to the attacks of wasps. These larvae suffered significantly higher mortality compared to caterpillars that were allowed to acclimate for longer periods of time.

Key Words: defensive behavior; herbivore; Lepidoptera; methodology; *Orgyia leucostigma*; predation risk

Resumen

Existen inconvenientes asociados con los métodos utilizados actualmente para cuantificar la depredación de insectos. Sin embargo, ha habido relativamente poco esfuerzo para documentar experimentalmente las fuentes de sesgos asociados con las diferentes metodologías. En este estudio, investigamos cómo los métodos utilizados para cuantificar experimentalmente la depredación pueden influir en las defensas antidepredadoras de insectos y, por lo tanto, alterar el número de presas muertas por los depredadores y potencialmente las conclusiones reportadas en el estudio. Nos centramos en el periodo de aclimatación, que representa el tiempo entre el momento en el que una presa está disponible, por ejemplo, en una hoja, y el inicio del experimento. En esencia, éste es el periodo de tiempo durante el cual un organismo se ajusta a las condiciones que lo rodean antes de que se imponga un tratamiento. Investigamos si el periodo de aclimatación de las presas en las plantas influye en el comportamiento antidepredador de las larvas de *Orgyia leucostigma* (J.E. Smith) (Lepidoptera: Lymantriidae), y el número de presas muertas por la avispa depredadora *Polistes fuscatus* (Fabricius) (Hymenoptera: Vespidae) en el laboratorio. Los resultados de este estudio proporcionan evidencia de que el tiempo de aclimatación afecta significativamente el comportamiento antidepredador observado y la mortalidad de las orugas expuestas a los depredadores. La mayoría de las larvas a las que se les permitieron periodos cortos de aclimatación no se tiraron de la hoja en respuesta a los ataques de las avispas. Estas larvas sufrieron una mortalidad significativamente mayor en comparación con las orugas a las que se les permitió aclimatarse durante periodos de tiempo más largos.

Palabras Clave: comportamiento defensivo; herbívoro; Lepidoptera; metodología; *Orgyia leucostigma*; riesgo de depredación

Quantifying insect predation is difficult because often prey and predators can be very small, prey may be concealed, and predator-prey interactions can be relatively brief. In addition, there are few if any remains of the prey that can be detected after a predation event, other than in the gut or excreta using molecular tools (Symondson 2002). Given the degree of difficulty of assessing predation in the field, several methods have been designed for estimating predation (reviewed by Luck et al. 1988; Sunderland 1988; Kidd & Jervis 2005; Furlong & Zalucki 2010). One of the most frequently used approaches for studying the impact of predators on insect prey has been the use of arenas in the laboratory, where insect mortality is measured through direct observations or by the quantification of the number of prey killed per unit time (Kiritani & Dempster 1973; Grant & Shepard 1984; Furlong & Zalucki 2010). The use of arenas in the laboratory are particularly critical in the development of classical biological control programs using natural enemies because non-target testing needs to be done in

controlled environments before a natural enemy is released (Culshaw-Maurer et al. 2020).

Several studies have addressed some of the potential disadvantages and biases that can result when insect predation is estimated with methods such as laboratory arenas (Kiritani & Dempster 1973; Grant & Shepard 1984; Kidd & Jervis 2005). However, there has been relatively little effort to experimentally document the sources of biases associated with different insect predation detection and quantification methods. Higher estimates of prey mortality usually are obtained using laboratory arenas compared to field estimates, due in part to predators encountering prey more often in laboratory arenas than would be expected in the field (Kiritani & Dempster 1973; Luck et al. 1988; Macfadyen et al. 2015). The type and size of a study arena used to assess predation can influence the results obtained from the experiments, with predation rates usually decreasing from small, closed containers to open containers to plants within cages (Macfadyen et al. 2015). The

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placement of prey can influence predation results in artificial arenas, because predator searching behavior and efficiency may change on different plant parts and species (Coll et al. 1997; Castellanos et al. 2010; Barreto-Barriga et al. 2021). Food deprivation also can influence rates of predatory attacks, and in ambush predators the attack distance increases with hunger (Wallin & Ekblom 1994; Taylor & Schmidt 1996). The use of sentinel prey to estimate predation also can influence predation results because predators differentially respond to sentinel prey items, and different sentinel prey types vary in their effectiveness (Greenop et al. 2019; Boetzel et al. 2020; Nagy et al. 2020).

Laboratory arenas may alter prey behavior (Sunderland 1988), which may in turn affect and influence susceptibility to predation. For example, caterpillar response and escape from predators is influenced by how long they are in a container before the predator is introduced (I Castellanos, personal observation). This prey acclimation to laboratory settings after being manipulated rarely is acknowledged when predation is quantified. Thus, there is a need for investigating how quantification methods can affect insect antipredator defenses and alter estimates of predation.

Larvae of *Orgyia leucostigma* (J.E. Smith) (Lepidoptera: Lymantriidae) are polyphagous and external solitary feeders primarily on the foliage of deciduous trees (Raffa & Powell 2004), and are subject to predation principally by birds and flying invertebrate predators (Medina & Barbosa 2002). The vespine wasp, *Polistes fuscatus* (Fabricius) (Hymenoptera: Vespidae) is a major predator of caterpillars, which they capture, process, and feed to their larvae (Gould & Jeanne 1984). *Orgyia leucostigma* larvae do not spin a silk matt but are covered with conspicuous defensive hairs (Payne 1917), which act as physical barriers that deter invertebrate predators and provide sensory inputs that trigger defensive behavior that reduces predation (Castellanos et al. 2011a). *Orgyia leucostigma* caterpillars escape from *P. fuscatus* wasps by dropping from the host plant without laying down silk (Castellanos et al. 2011a). If they reach the ground when dropping, they return to host plants by walking on the leaf litter where they can be subjected to ground predation (Castellanos et al. 2011b).

In this study we investigated how predation quantification methods may influence insect antipredator defenses and alter estimates of predation. To do this, we examined how prey acclimation period on plants influences antipredator behavior of *O. leucostigma* caterpillars when they are exposed to the predator *P. fuscatus*, and if differences in acclimation periods result in different number of prey killed by predators.

Materials and Methods

STUDY ORGANISMS

Orgyia leucostigma caterpillars used in the experiments originated from a laboratory colony established using larvae collected from *Acer negundo* L. (Aceraceae) (box elder) trees in Patuxent Research Refuge (39.041666°N, 76.791666°W), Laurel, Maryland, USA. Larvae eclosing from egg masses were reared individually in 437 mL plastic deli containers in the laboratory at room temperature (25 ± 2 °C) and fed *A. negundo* foliage. *Polistes fuscatus* have been identified as a common member of the insect community in tree canopies in Patuxent Research Refuge (P Barbosa, I Castellanos, AE Segarra, unpublished data), and have been observed attacking and consuming *O. leucostigma* caterpillars at Patuxent Research Refuge (P Barbosa, personal observation). Wasps used in this experiment were obtained from nests collected in Patuxent Research Refuge. Colonies were maintained in the laboratory at room temperature (25 ± 2 °C) and were provided daily with equal volumes of fresh water, honey, and geometrid, lymantriid, and tenebrionid larvae.

EXPERIMENTAL PROTOCOL

To determine if prey acclimation period on plants influences prey antipredator behavior and mortality, 1 d old third instar *O. leucostigma* were acclimated before being exposed to *P. fuscatus*. For each trial, a caterpillar was placed on a leaf of a 30 cm *A. negundo* branch positioned 0.5 m above the ground for different periods of time, and afterwards the branch was introduced into a 1.0 × 1.0 × 1.5 m cage containing a wasp nest. Individual branches were introduced inside cages with wasps that had been previously conditioned to fly from their nest to the experimental leaf as soon as the branch was introduced. All 30 cm branches had approximately the same number of leaves without leaf damage and were obtained from the same distance from the canopy. Caterpillars were placed on a leaf of each branch by carefully sliding the leaf underneath a motionless caterpillar. Single branches with caterpillars were introduced inside the cage at time periods that varied from 0 to 60 min acclimation. A different box elder branch and caterpillar were used for each trial ($N = 105$). Sixty female wasps from 6 different nests were used, and a wasp individual was not used in a consecutive trial nor used for at least 3 d after a trial. Wasps were marked individually with colored nontoxic ink to avoid using wasps in consecutive trials. To control for wasp hunger, experimental wasps were fed the same diet and then starved for 24 h before they contacted a caterpillar. Only single wasps that flew from their nest to the experimental leaf were used in the experiments. Multiple wasps flew to the experimental branch in less than 10% of the trials and these were excluded from the analysis.

The behavioral responses of caterpillars to wasps and the number of predated caterpillars were recorded. A caterpillar was recorded as a survivor if it escaped the attack from a wasp, which occurred by dropping to the ground in response to contact with a predator.

STATISTICAL ANALYSES

The acclimation times, which varied from 0 to 60 min were grouped into 3 categories. These categories were: less than 20 min (designated as 20 min, $N = 43$, average acclimation time = 5.3 min, minimum acclimation time = 1 min, maximum acclimation time = 15.5 min); from 20 to < 40 min (designated as 40 min, $N = 26$, average acclimation time = 32.7 min, minimum acclimation time = 23.1 min, maximum acclimation time = 39.5 min); and from 40 to 60 min (designated as 60 min, $N = 36$, average acclimation time = 46.5 min, minimum acclimation time = 40 min, maximum acclimation time = 60 min). These time periods were selected because in preliminary observations we had noticed that caterpillars were able to respond by dropping in response to a wasp contact after 20 to 40 min of being placed on the leaf of a branch.

Caterpillar mortalities in the different acclimation categories were analyzed using chi-square tests of independence. The frequencies of different behavioral responses of caterpillars in the acclimation experiments (drop from leaf and no response) were compared among treatments using chi-square tests of independence. The family-wise error rate for multiple comparisons was controlled using a Bonferroni correction (Sokal & Rohlf 1995).

Results

Caterpillars did not feed during the experiments, and all were found and contacted by wasps. Mortality of larval *O. leucostigma* was influenced significantly by acclimation period ($\chi^2 = 11.65$; $df = 2$; $P = 0.003$) (Fig. 1). When exposed to foraging wasps, the mortality of caterpillars that acclimated on the plant between 20 and < 40 min (35%) or between 40 and 60 min (42%) was significantly lower than the mortality of caterpillars that had less than 20 min to acclimate (72%) ($\chi^2 =$

7.87; $df = 1$; $P = 0.015$; and $\chi^2 = 6.26$; $df = 1$; $P = 0.036$, respectively). No difference in mortality was found between caterpillars that acclimated between 20 and < 40 min and those acclimated between 40 and 60 min ($\chi^2 = 0.09$; $df = 1$; $P = 0.765$) (Fig. 1).

Caterpillars predominantly dropped off the plant in response to predators when acclimated between 20 and < 40 min (65.4%) and between 40 and 60 min (58.3%), and the majority did not respond when their acclimation period was less than 20 min (72.1%) ($\chi^2 = 11.64$; $df = 2$; $P = 0.003$) (Fig. 2). The percentage of caterpillars that dropped in response to wasps that were acclimated for less than 20 min (27.9%) was significantly smaller than the percentage of caterpillars that were acclimated between 20 and < 40 min (65.4%) or between 40 and 60 min (58.3%) ($\chi^2 = 9.34$; $df = 1$; $P = 0.006$ and $\chi^2 = 7.46$; $df = 1$; $P = 0.018$, respectively) (Fig. 2). No difference in the percentage of dropping was found between caterpillars that acclimated on the plant between 20 and < 40 min and those acclimated between 40 and 60 min ($\chi^2 = 0.32$; $df = 1$; $P = 1$).

Discussion

Despite the risk of laboratory arenas altering prey behavior, we were able to provide the conditions that allowed the anti-predator behavior (dropping-off the leaf) to occur for this predator-prey interaction. Dropping off a plant may be the most common evasive behavior found in exophytic insects (Witz 1989; Gross 1993; Greeney et al. 2012), and the results of this study provide evidence that acclimation time significantly affects this behavioral response which enhances survival. The majority of larvae with acclimation periods of less than 20 min did not drop in response to the attacks of wasps and suffered significantly higher mortality compared to caterpillars that were allowed to acclimate for more than 20 min. However, we currently do not have field larval mortality data to determine if the 32 to 42% mortality of acclimated caterpillars found in our study reflects field survival.

Two possible explanations may account for the decrease of dropping behavior in caterpillars with shorter acclimation periods. Larval *O. leucostigma* may have been easier to detect by wasps during the first 20 min after being placed on a leaf because caterpillars walked and stopped several times on the underside of leaves before they remained motionless (I Castellanos, personal observation). It is likely that during this initial period, caterpillars are searching for sites under leaves where they are less apparent to predators, because species of this genus rely on being cryptic to escape predation (Sandre et al. 2007). If non-acclimated caterpillars were easier to detect due to their wandering, wasps could have perceived them from a longer distance, which in

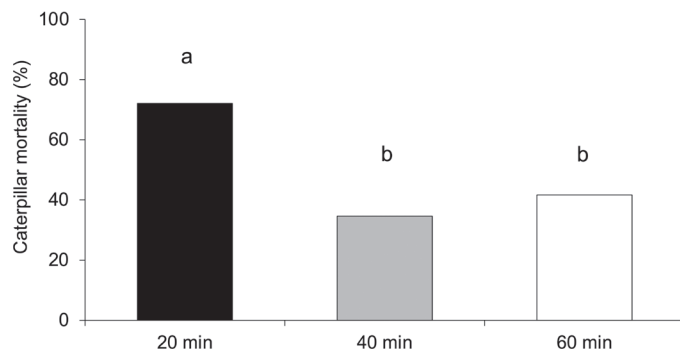


Fig. 1. The mortality of *Orgyia leucostigma* caterpillars that acclimated on the plant for less than 20 min (20 min), from 20 to < 40 min (40 min), and from 40 to 60 min (60 min) before exposure to *Polistes fuscatus* wasps. Treatments with the same letter are not significantly different ($P > 0.05$).

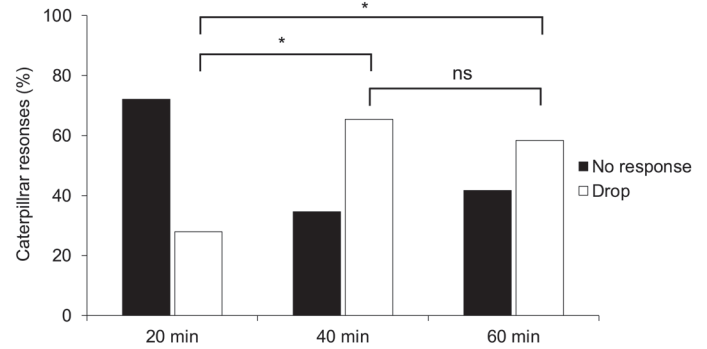


Fig. 2. Behavioral responses of *Orgyia leucostigma* caterpillars that acclimated on the plant for less than 20 min (20 min), from 20 to < 40 min (40 min), and from 40 to 60 min (60 min) to the attacks of *Polistes fuscatus* wasps. * $P < 0.05$; ns: $P > 0.05$. No response = caterpillars that did not respond to predators by dropping. Drop = caterpillars that dropped from the leaf in response to the predator.

turn will have increased attacking speed allowing wasps to reach and bite a caterpillar's body before it was able to drop (Castellanos et al. 2011b).

A second explanation for this pattern is that predatory stimuli do not trigger dropping behavior during the first min a caterpillar has been placed on a leaf. Dropping behavior in *O. leucostigma* caterpillars occurs when predators bend their hairs with high velocities (Castellanos et al. 2011a). Preliminary experiments showed that *O. leucostigma* caterpillars do not drop in response to a controlled stimulus that consists of displacing their dorsal hairs with high bending velocities during the first 10 min after being placed on a leaf, but do respond by dropping if the stimulus is applied after 20 min of being placed on a leaf. Previous studies have reported that antipredator behaviors are triggered only under particular prey behavioral contexts (Hoy et al. 1989; Cinel et al. 2020). For example, sensory neurons responsible for eliciting escape responses in crickets in response to insectivorous bats are triggered only in the appropriate behavioral context, when an individual performs flight behavior (Nolen & Hoy 1984). Thus, it is likely that dropping behavior in larvae of *O. leucostigma* is triggered only after individuals reach a particular site and remain motionless on the underside of a leaf, where they are less apparent to predators (Sandre et al. 2007) and are able to respond appropriately to the attack of a predator (Hoy et al. 1989; Cinel et al. 2020).

It has been recognized that there are limitations in the use of current laboratory and field methodologies to detect and quantify insect predation, and that laboratory mortality data can indicate only the relative predation potential of the insect prey species, which may not be realized in the field (Macfadyen et al. 2015). However, there has been relatively little effort to experimentally document the sources of biases inherent in different insect predation detection and quantification methods (Castellanos et al. 2015). The results of this study show that the method used to quantify insect predation can influence insect antipredator defenses, which can be the cause of overestimating mortality in the laboratory. Thus, estimating the potential impact that individual predators may have on prey species should involve careful planning of arenas and experimental methods to include an understating of the behavioral responses of prey to predators.

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