The presence of active larvae delays the emergence of conspecifics in the tupelo leafminer, Antispila nysaefoliella

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ABSTRACT

Hypothesis: In many systems, density-dependent effects on fitness often can be mitigated with adaptations such as individual mobility or increased competitive ability. However, in sessile or sedentary systems, immobility might preclude individuals from directly choosing their group or patch, thus making it difficult for them to respond to the costs and benefits of group living or variation in density in general. In this study, I test the effect of density on delayed emergence of an effectively sessile species.

Study system: Tupelo leafminer, Antispila nysaefoliella Clemens (Lepidoptera: Heliozelidae), a specialist of black gum, Nyssa sylvatica Marsh (Cornaceae). The study population is located in the northern Shenandoah Valley in Clarke Co., Virginia.

Methods: In 2004 and 2008, I conducted field experiments where I reduced the initial density of larvae on leaves and compared the frequency of occurrence of new larvae after the experimental treatment. In 2008, I also enclosed half of the leaves with fine-mesh bags to exclude compensatory oviposition as a possible cause of subsequent larval emergence.

Prediction and result: The prediction that the removal of active larvae would lead to more occurrences of secondary larval emergence on experimental leaves compared with control leaves was supported in both years of the experiment. The mesh enclosure treatment (2008) had no effect, which suggests that the higher rate of secondary emergence was not due to eggs that were deposited after larvae were removed.

Conclusion: The experimental results demonstrate that the presence of active larvae may suppress the development of conspecific larvae (or unhatched eggs). Therefore, the potential number of A. nysaefoliella larvae on leaves might not be realized immediately at the beginning of the season. Rather, as individuals in the initial cohort die, typically due to parasitism, others may emerge subsequently. Although the mechanism is currently unknown, delayed emergence appears to be associated with larval density.

Keywords: aggregation, competition, delayed hatching, density dependence, hatching asynchrony, life-history timing, plant–insect interactions.

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INTRODUCTION

Animal aggregations and evidence of their benefits are widespread, and one of the most commonly reported benefits is a reduction in predation risk through the effects of numerical dilution, predator confusion or collective vigilance (Clark and Mangel, 1986; Roberts, 1996; Prokopy and Roitberg, 2001; Krause and Ruxton, 2002). Aggregation can also facilitate feeding, increase the rate of food finding or improve thermoregulatory abilities and subsequent physiological responses for nutrient assimilation (Brown, 1986; Elgar, 1986; Stamp and Bowers, 1990; Denno and Benrey, 1997; Beauchamp, 1998; Fordyce, 2003; Reader and Hochuli, 2003). However, the potential benefits gained from grouping depend largely on the given environmental and social conditions, and thus may diminish or fluctuate with changes in predation risk, density, resource quality or availability (Hamilton, 1964, 1971; Parrish and Edelstein-Keshet, 1999; Prokopy and Roitberg, 2001).

To meet such changing demands, individual mobility allows for nearly immediate responses to variable conditions, and gives rise to the expectation that group size or the spatial position of individuals within a group is dynamic and can be achieved through joining or avoidance behaviours of adaptively behaving individuals (Parrish and Edelstein-Keshet, 1999; Prokopy and Roitberg, 2001). This concept has motivated a wealth of studies, both theoretical and empirical, to understand the evolution of behavioural strategies and their effects on group dynamics (Hamilton, 1971; Caraco and Wolf, 1975; Clark and Mangel, 1986; Elgar, 1986; Turner and Pitcher, 1986; Godfray et al., 1991; Wrona and Dixon, 1991; Krause, 1994; Parrish and Edelstein-Keshet, 1999; Krause and Ruxton, 2002; Peacor, 2003). However, in many systems, individuals are not freely mobile, and group membership is largely determined by chance of settlement [e.g. barnacles, corals (Carlon and Olson, 1993; Bertness et al., 1996)], resource patchiness [e.g. fruit, dung, carrion flies (Woodcock et al., 2002; Takahashi, 2007)] or the oviposition decisions of their mothers [e.g. larvae of herbivorous insects, parasitic wasps (Stamp, 1980; Damman, 1991; Edgerly et al., 1998; Gribenberg et al., 2007; Randikofler et al., 2007)]. In addition, if further recruitment is limited or unlikely, the loss of group members can lead to consequences for the surviving members – if there are indeed benefits derived from being in a group. Therefore, immobility can set the stage for the evolution of traits and behavioural strategies that allow for relatively rapid responses to the changing demands of group living and other density-dependent effects.

Leaf-mining larvae are a good example of sessile organisms that have little control over their location and choice of their natal leaf. Nevertheless, their survival and reproductive success are linked intimately with the leaf upon which they feed and grow (Faeth, 1990, 1991; Connor and Taverner, 1997; Cornelissen and Stiling, 2006). Moreover, the number of larvae on a leaf can directly influence parasitism risk and the amount of food resources per capita (Connor and Cargain, 1994; Inouye and Johnson, 2005; Low, 2008; Low et al., 2009). In one leaf-mining species, Antispila nysaefoliella Clemens (Lepidoptera: Heliozelidae), it has been reported previously that leaves with more larvae were (1) more detectable to parasitoids, but incurred lower per capita parasitism, and (2) feeding rates were poorer at low and high densities (Low, 2008; Low et al., 2009). How are the density-related consequences to fitness mitigated in A. nysaefoliella?

From 2001 to 2008, I had observed that the initial emergence of A. nysaefoliella larvae occurred synchronously and approximately on the same date each year. Thereafter, however, larvae would appear in a much more staggered sequence and much less synchronously (on the same leaves). Because the timing of emergence determines the effective group size of an individual, it is likely to have direct and important effects on the fitness functions of A. nysaefoliella. Similarly, as in many other systems, emergence time can be a critical
determinant of both survival and reproductive fitness (Moore and Sih, 1996; Edgerly et al., 1998; Clauss and Venable, 2000; Morbey and Ydenberg, 2003; While et al., 2009).

This paper is a first step towards understanding the potential cues that may be associated with delayed emergence in *A. nysaefoliella*. In this study, I conducted two years of field experiments to test if delayed larval emergence is caused by the presence of actively feeding larvae. Here, I report the experimental results and discuss some possible underlying mechanisms for the observed effects.

**METHODS**

**Natural history**

*Antispila nysaefoliella* is a leaf-mining moth that specializes on the leaves of black gum, *Nyssa sylvatica* Marsh (Cornaceae), which is distributed throughout the southeastern United States. The study site is located in a mixed deciduous forest in the northern Shenandoah Valley, Virginia. In early spring of each year, soon after the leaves of *N. sylvatica* have flushed, adults emerge and persist in high abundance for approximately 3–4 weeks. During this time, adults can be found very easily by sight resting on the undersides of leaves or moving between leaves and branches throughout the day. Oviposition occurs during the day and is most active during warmer temperatures. Observation indicates that eggs deposited in May might lay dormant on leaves for several months before hatching into larvae. Multiple females will search and oviposit on the same leaves, and larval density can range from 1 to 48 larvae per leaf and from 0.02 to 0.8 larvae per cm$^2$ leaf area (C. Low, unpublished data 2001–2008). Larvae emerge nearly synchronously in the fall, typically around the first of August of each year. Thereafter, the population is marked by staggered emergence times, and larvae can be found until complete leaf fall, which typically occurs in late October.

In general, after the egg hatches, a larva burrows into the mesophyll and creates a mine through its feeding and tunnelling activity (hence, ‘mining’), and continues to feed and grow in isolation. This feeding causes the mine to expand and to become more visually apparent; and because *A. nysaefoliella* feeds in both upper and lower leaf layers of the mesophyll, their mine becomes a highly visible semi-transparent blotch-shaped ‘window’ where the larva can be found easily from simple visual inspection (Fig. 1) (Hering, 1951; Johnson and Lyon, 1991). The mines of *A. nysaefoliella* always begin at a leaf vein, expand radially, and become more oblong-shaped at later instars (Fig. 1). Given that mines are often more apparent than the larvae themselves, they are useful for detecting the presence of larvae, and are good visual proxies of larval size and developmental stage.

**Larval removal experiments**

The objective of the following experiments was to determine if the presence of active larvae delays the emergence of conspecific larvae. With individual leaves serving as the experimental units, I compared the proportion of leaves with secondary larval emergence between leaves where larvae were experimentally removed (‘treatment’) and unmanipulated (‘control’). In 2004, one larva was randomly chosen to remain on the experimental leaves to match the minimum of one larva on control leaves. In contrast, in 2008, all larvae on an experimental leaf were removed. I removed larvae by piercing the mine epidermis using...
superfine-tipped stainless steel forceps (Rubis Forceps #4524, BioQuip Products, Inc., Rancho Dominguez, CA, USA) and extracting them from their mines. This procedure did not damage the active leaf tissue.

On 20 August 2004, when the first mines of the season became apparent (≈1 mm in diameter), leaves with group sizes of 1, 2–4, 5–9, and ≤10 mines per leaf were selected from the lower canopy layer of each of four trees using a 3-m ladder. For each category of group size, the number of leaves selected is given in parentheses (ncontrol, ncontrol): 1 (23, 0), 2–4 (13, 27), 5–9 (14, 18), and ≤10 (14, 18). The identity and development of individual mines (i.e. larvae) were tracked by comparing series of digital images taken of each leaf on nine dates (20 August to 13 September 2004). The survival status of each larva was assessed on 20 August and 26 August. However, to confirm that an individual larva was no longer feeding and no longer alive, the full sequence of images taken over the entire development period (up to ≈24 days) was compared. With this method, mortality that had occurred during the experimental period (6 days) could be confirmed by the subsequent lack of development (i.e. no mine expansion) by the end of the season. Another visual indicator that a larva has died is necrosis around the mine edge. This occurs because there is no longer any active feeding to expose fresh leaf tissue.

**Fig. 1.** Experimental leaves with mines are pictured. The leaf on the right (#60C ‘control’) has mines with visible larvae inside, and the leaf on the left (#60T, ‘treatment’) has empty mines from which the larvae have been removed.
I repeated this experiment on 23 August 2008, using the methods described above. However, in 2008, I enclosed approximately half of the total leaves \((N = 143)\) individually in small fine-mesh bags to exclude the possibility of compensatory oviposition as the cause for the results observed in 2004. In 2008, all larvae on experimental leaves were removed to clearly differentiate new mines from pre-existing mines. In addition, because this experiment was targeted at excluding oviposition and replicating the effects of larval removal, mine development was not monitored with photographs over time, but taken only on the day of removal and 14 days later on 6 September 2008. New mines could be easily detected by comparing the two sets of images. Leaves ranged from 1 to 28 larvae per leaf in the 2008 study, and the number of leaves for each group size category are as follows [group size \((n_{\text{control}}, n_{\text{treatment}})\): 1 (11, 10), 2–4 (19, 21), 5–9 (19, 24), and \(\leq 10\) (17, 22). Approximately half of these were enclosed in the mesh bags: 1 (4, 4), 2–4 (9, 9), 5–9 (11, 16), and \(\leq 10\) (11, 10).

The difference in the number of leaves that resulted in new mines between control and experimental leaves was tested using Fisher’s exact test (Sokal and Rohlf, 1995). The relationship between the number of new mines initiated and the number of larvae removed was evaluated using linear regression; and the difference between the slopes of the resulting regression functions of the experimental and control leaves was evaluated using the slope test (Sokal and Rohlf, 1995). Leaf size was also measured using image analysis software (Sigma Scan 5.0) and entered as a covariate in the regression analysis because of its possible correlation with larval or egg density.

**RESULTS**

In 2004, new larvae emerged more frequently on leaves where larvae were removed experimentally than on control leaves (Fisher’s exact test, \(P = 0.015\); Fig. 2). In addition, the number of newly emerged larvae \((y)\) could be predicted by the number of larvae removed \((x)\), both experimentally \((y_T = -0.62 + 0.68x, R^2 = 0.39, \text{d.f.} = 69, P < 0.001)\) and naturally \((y_C = -0.08 + 0.42x, R^2 = 0.41, \text{d.f.} = 61, P < 0.001)\). The slope of this relationship was significantly greater for experimental leaves compared to control leaves (slope test, \(t_{128} = 4.35, P < 0.001\)). Leaf size did not have an effect (control \(P = 0.37\), treatment \(P = 0.40\)).

In 2008, larvae also emerged more frequently on experimental leaves than on control leaves (Fisher’s exact test, \(P < 0.001\) for both enclosed and un-enclosed leaves; Fig. 2). Enclosed and un-enclosed leaves did not differ (i.e. both resulted in significant positive treatment effects at \(P < 0.001\)), and the data were pooled in the final analysis. As in 2004, the number of new mines \(y\) could be predicted by the number removed \(x\) \((y = 1.0 + 0.11x, R^2 = 0.132, \text{d.f.} = 75, P = 0.001)\), which was analysed using only the data from the experimental leaves since occurrences of mortality were not confirmed for control leaves in this experiment. Therefore, the number of larvae that were naturally removed \((x)\) would be inaccurate. The direction and magnitude of effects were also similar to the 2004 results (Fig. 2).

**DISCUSSION**

In this study, the experimental removal of existing larvae led to the emergence of new larvae on the same leaves. This result suggests that the presence of active leaf-mining larvae may inhibit, or otherwise delay, the emergence of conspecific larvae. First, because the
experimental treatments occurred at the very early stages of larval development, all leaves had nearly the maximum of potential available space for all larvae to develop simultaneously at the start of the season. However, there were many pre-existing eggs that clearly had not yet hatched. Second, if the staggered emergence times were related to differences in egg development or dormancy time, then the experimental removal of larvae would not have led to a treatment effect and both control and treatment leaves would have resulted in the same rate of new emergences. Third, even though cannibalism may occur naturally, it does not explain the experimental results or the observation that there are staggered emergence times in this system. Fourth, the additional mesh enclosure treatment in the 2008 experiment provided clear evidence that more recent oviposition events were not responsible for the subsequent emergences of larvae. Finally, I have also observed that larvae commonly appear in the available spaces on leaves soon after pupation of earlier larvae – indicating that there are pre-existing eggs that are dormant until the activity of earlier larvae ceases.

Very little is currently known about the mechanisms that can regulate the interaction between different life stages of herbivorous insects that are temporally separated but share the same resource. One possible mechanism may be chemical mediation via the host plant. If there is a chemical signal (direct or indirect) that is induced by actively feeding larvae on leaves, then this may act to suppress egg or larval development of congeners. In *A. nysaefoliella*, eggs are deposited near leaf veins on the undersurface of leaves, and

![Graph](image-url)

**Fig. 2.** The proportion of leaves that resulted in newly emerged larvae after the experimental removal of *A. nysaefoliella* larvae from their mines. Larvae were removed on 20 August 2004 and on 23 August 2008; and the responses were measured 6 days later on 26 August and 14 days later on 6 September, respectively. In 2004, the number of larvae on experimental leaves was reduced to a single individual. In 2008, all larvae were removed. Dark bars represent the control leaves where no larvae were removed; lighter bars are leaves where larvae were removed. In 2008, approximately half of the leaves were enclosed in fine-mesh bags to prevent the possibility of further oviposition ('2008-X'). Numbers within the bars indicate sample size (number of leaves). *P* < 0.05, ***P* < 0.001.
damage to the vascular tissue from egg deposition is most likely to be less severe than when larvae actually feed (Hilker and Meiners, 2002, 2006). Thus, it may be only when mining begins that plant defences are elicited. Furthermore, the period of about 2–3 months between oviposition and mine development suggests that eggs can remain on the leaf without consequence. In general, the chemical changes that may be induced by oviposition, egg attachment or herbivory are not necessarily specific to the insect life-history stage per se, but to the particular plant tissue that is damaged or the specific symbionts that are present (Hilker and Meiners, 2002, 2006). Larval feeding may be the first cue that induces a defensive response by the host plant.

The positive association between the number of larvae removed and the number that emerge subsequently may be consistent with a plant-mediated effect. The weaker positive association on control leaves compared with experimental leaves may be due to a weaker chemical signal given that larvae often can continue to feed and grow after being parasitized or suffering another affliction (Godfray, 1994). In comparison, on experimental leaves, larvae were removed instantaneously, so that any potential inhibitory cues that may be caused by active larval feeding would have stopped more quickly. Alternatively, cannibalism of eggs by existing larvae could be contributing to the observed weaker association between the number of new larvae and number removed on the control leaves. This will be investigated in a future study.

In A. nysaeofoliella, the variable emergence times of larvae may be an adaptive response to the temporal variation in parasitism risk by affecting not only the timing per se, but also the group size dynamics and the associated benefits to survival. The data from a molecular analysis of parasitism indicate that ~60% of the total parasitism occurs within the first few days of the season and to first instar larvae (C. Low et al., in preparation). If this variation were consistent across years, then having temporally separated emergence times would provide an advantage directly. However, because time and resources are limited, early emergence allows for the maximum amount of resources to be available, and emerging too late in the season would be disadvantageous in the absence of parasitism. Therefore, emerging soon after parasitism has already occurred to minimize potential risk could be the optimal time to hatch and begin feeding.

Many studies have shown that the timing of birth has a direct relationship with competitive hierarchies and setting social environments, which can influence both performance and survival functions (Benrey and Denno, 1997; Edgerly et al., 1998; Low et al., 2009; While et al., 2009). In A. nysaeofoliella, whether it is in the interest of the early larvae to suppress potential competitors while reaping the benefits of group living or the late larvae to avoid parasitism but risk lower resource availability is an open question. This is simply one question among many about this intriguing system and much more research is needed to understand the mechanisms that underlie the interactions, behaviours, and evolutionary ecology pertaining to delayed emergence in A. nysaeofoliella.

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