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Article

Effect of a Marking Pheromone and Population Density on Ladybird Larval Development

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Simple Summary

Larvae of ladybird beetles living in a high number together can compete for food and cannibalize other larvae. They can feel their density as number of encounters and smell their footprints. We reared larvae of Harlequin ladybird *Harmonia axyridis* in three densities (1, 4, 8 in a dish) and either in dishes cleaned daily or with accumulated smell. We measured the time of development of larvae and the body mass of the adults subsequently emerged. Larvae in dirty dishes kept developing fast even at high density, while larvae in clean dishes developed slowly at the high density. They thus avoided becoming defenseless pupae in presence of other feeding larvae. Adult beetles that resulted from larvae living at high density were much lighter than at low density. This effect was probably caused by the frequency of meeting other larvae in a dish, not by the amount of the chemicals in footprints, because beetles from dirty dishes were slightly heavier. High-density populations may begin to self-regulate due to negative effects on growth and survival, reducing the risk of overpopulation.

Abstract

Females of predaceous ladybirds use sensing chemicals in larval tracks as oviposition deterring pheromone to avoid cannibalism of eggs. We hypothesized that larvae would also respond to the presence of conspecific tracks by slowing their developmental rate and delaying pupation, thereby reducing the time spent as a defenseless pupa in the presence of feeding conspecifics. We reared larvae of the harlequin ladybird *Harmonia axyridis* in dishes that were replaced daily by a clean one (C) or continuously in a dish with larval tracks accumulated (P). We used three larval densities (1, 4, 8 larvae per dish) for both treatments (C1, C4, C8, P1, P4, P8). We measured the developmental time of the fourth larval instar and fresh adult body mass. Developmental time increased at the highest density in the clean treatment C8 but remained unchanged across densities in the pheromone treatment (P1-P8). Body mass was significantly lower at the highest density in both treatments (C8, P8) and was slightly higher at the presence of pheromone (P). Ladybird larvae respond independently to their density and to the presence of pheromones. The compounds present in the tracks, previously known as oviposition deterring pheromone, may therefore also function as development enhancing pheromones.

Keywords: semiochemicals; Coccinellidae; alkanes; hydrocarbons; population density

1. Introduction

The beetle family Coccinellidae (Coleoptera) is globally recognized for its striking colors but is also known worldwide for its significant impact on ecosystems and its role in pest control, sparking great interest both scientifically and in agro-industrial applications [1]. Coccinellids are natural predators, especially of aphids and scale insects, although some species are phytophagous or

mycophagous [2]. Many species exhibit complex trophic interactions, including intraguild predation (IGP) [3] and intra-specific cannibalism [4].

Reproduction begins once the adult female lays her eggs, in the tribe Coccinellini usually in clusters near a colony of aphids or other food source [5]. The eggs are bright yellow or orange and hatch within three to seven days, primarily depending on the temperature to which they are exposed [6]. The larvae go through four larval instars before reaching the pupal stage. Both eggs and pupae are immobile, do not actively defend themselves, and they are thus more prone to be cannibalized than larvae [7]. Older ladybird larvae may also attack and feed on smaller larval instars [8]. Adults and larvae of *Coleomegilla maculata* and *Propylea quatuordecimpunctata* attacked eggs, 1st and 2nd instars of *H. axyridis* [9]. It may seem that a selection for faster growth and development will minimize the risk of being cannibalized as an egg or a small larva, but it can increase the risk of being cannibalized as a pupa by slower conspecific 4th instar larvae.

Besides cannibalism, simple competition for food can be a driver for faster development. But this competition will be strong only when food is scarce, and thus the fast development would lead to smaller adults.

One form of intraspecific communication detected in insects provides protection of eggs and young larvae against cannibalism and IGP by older larvae or simply against competition for limited food source. It is known as the Oviposition-detering pheromone (ODP) or allomone. ODP in fruit flies consists of chemicals released by adult individuals that affect female decisions regarding egg-laying, leading other females to avoid depositing eggs in the same fruit [10].

Similar effects have been detected in aphidophagous predators *Coccinella septempunctata* and *Chrysopa oculata* [11]. In Coccinellidae, ODP are deposited by larvae. Females do not lay eggs or lay less eggs in presence of either conspecific or heterospecific larval tracks. This effect persists from one day to one month [12]. Chemical analyses of the compounds responsible for this communication included short-chain hydrocarbons and esters [13]. Initial studies on this phenomenon in the invasive harlequin ladybird, *Harmonia axyridis*, also demonstrated that chemical markers could deter females from laying eggs, thus reducing resource competition and cannibalism among larvae [14].

Population dynamics of systems with ladybirds and aphids can be better specified as a metapopulation model of aphid-ladybird interactions than by classical Lotka-Volterra models of predator-prey relationship [15]. Selection should favor mechanisms that enable predators to avoid reproducing in patches with insufficient prey and those already occupied by predators [16]. Optimization of the oviposition sites in systems with large generation times ratio (aphids having extremely short life cycle, compared e.g. to coccids [17]) then favor using compounds deposited by conspecifics as a clue to search for an alternative food patch [18].

Exposure of either larvae or adults of *Cheilomenes sexmaculatus* to IGP risk by *H. axyridis* decreased either adult or offspring body size [19]. As mentioned above, pupae are also in risk of IGP and cannibalism. We expect that ODP may also influence larval developmental rate and resulting body size. Larvae could either accelerate their development to avoid competition for food or to decelerate it not to become defenseless pupae in presence of voracious larvae.

Population density should be important in both competition and cannibalism; and truly, cannibalism increased with larval density in *Cycloneda sanguinea* and *H. axyridis*, suggesting that not all attacks on conspecifics are driven by hunger [20].

This research investigated whether exposure to larval tracks and the intensity of physical larval presence would affect the developmental time of *H. axyridis* larvae and body mass of resulting adults. The development times of the last larval instar and the pupal stage were measured in the presence of diverse amounts of larval tracks pheromone and at various population densities.

2. Materials and Methods

We maintain a laboratory stock of the ladybird *H. axyridis* named Stáňa, which belongs to the colour form *novemdecimsignata* (otherwise known as *succinea*) at 25°C, 16:8 LD [21]. We feed them with frozen *Ephestia* eggs and aphids *Acyrtosiphon pisum* reared on *Vicia faba* and provided them with

water in cotton pads and corrugated paper for oviposition. Eggs were separated from the adults and larvae were reared in groups of five in 10 cm diameter Petri dishes. Water and food were renewed once a day until the larvae reached the third instar. Then we checked them twice a day (once every 12 hours) until larvae reached the fourth instar. Then the larvae of the same developmental stage (within a 12-hour interval) were separated into new clean Petri dishes with sufficient water and food.

From the moment when larvae reached the fourth instar, they were separated into six treatments. Three treatments were so called "clean" (C) and three were called "pheromone" (P). The individuals of the clean treatments were moved to a new clean dish daily at the same time to avoid accumulation of pheromones. The larvae of the pheromone treatments remained in the same dish until pupation to allow accumulation of pheromones. In both, water was changed and frozen *Ephestia* eggs ad libitum were replenished each day to minimize cannibalism. Within these groups, three treatments were set up with different larval densities per 15 cm Petri dish: 1 larva living alone (C1, P1), 4 larvae living in a group (C4, P4), and 8 larvae living in a group (C8, P8).

The developmental times of the fourth instar and of the pupa were measured in 12-hour intervals and recorded for each individual. The experiment was replicated three times within two months, resulting in 354 larvae measured. Within one day after each adult emerged from pupa, the body mass was measured to the precision 0.1mg. A total of 324 adult individuals from all treatments were examined.

The measured variables were analyzed using two-way ANOVA and Fisher LSD tests; standard significant difference value of $p < 0.05$ was considered.

3. Results

3.1. Developmental time

Developmental times of the 4th instar highly differed among the six treatments (Figure 1). Larval developmental time was shorter in P than in the C treatments ($F_{1,348}=16.8$, $p < 0.00001$). High density of individuals resulted in longer larval development ($F_{1,348}=7.8$, $p=0.0005$), mainly in the C treatment, as shown by strong interaction ($F_{2,348}=12.2$, $p < 0.00001$). Developmental time in treatment C8 was longer than in all other treatments ($p < 0.00001$), development of C4 was longer than that of P4 ($p=0.006$), but it was not different from C1 ($p=0.06$). Such a pattern suggests that a high number of physical encounters between larvae in clean environment prolongs their development, decreasing the risk of predation in the pupal stage. But high amount of pheromones from larval tracks reverse the effect, keeping the development as fast as in solitary larvae.

There was no significant difference in the pupal developmental time between the six treatments ($F_{2,348}=1.40$, $p=0.23$), suggesting that neither group density nor pheromone action affected development time from pupa to adult. In all cases, the average time was 5.5 days.

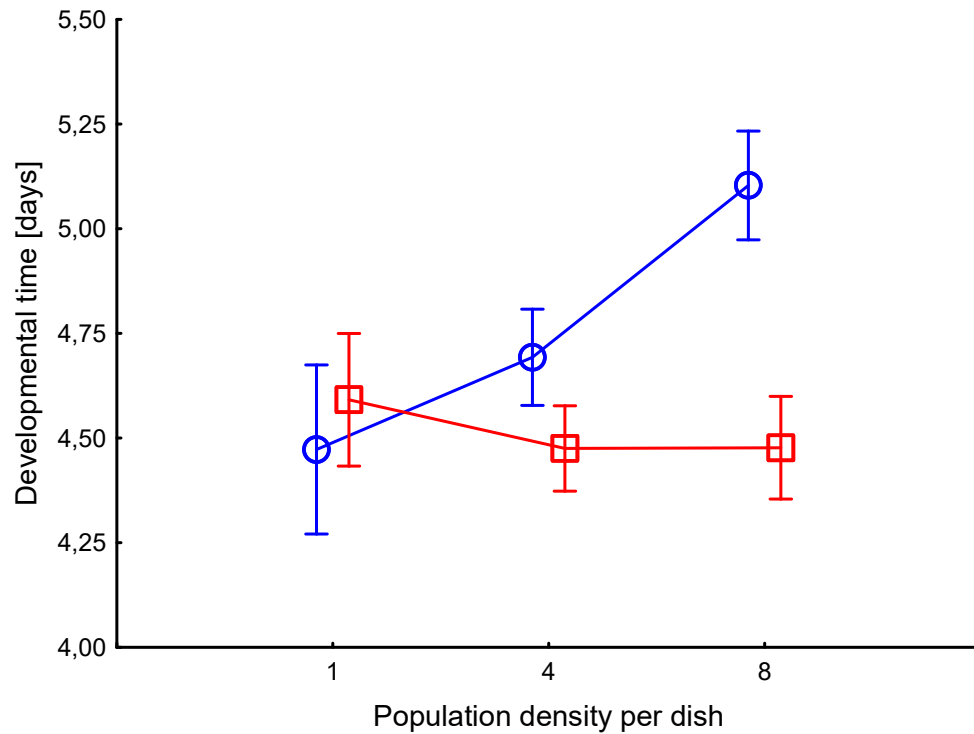


Figure 1. Developmental time of 4th instar larvae of *Harmonia axyridis* maintained in various numbers per 15 cm Petri dish and in Clean treatments (blue circles), and Pheromone treatments (red squares). Vertical bars denote 0,95 confidence intervals.

3.2. Adult body mass

Body mass of one day old adults highly differed among the six treatments (Figure 2) Body mass was higher in P than in C treatments ($F_{1,318}=5.7$, $p=0.017$). High density of individuals resulted in smaller adults ($F_{1,318}=20.7$, $p<0.00001$), similarly in C and P treatments (interaction $F_{2,318}=0.21$, $p=0.81$). Body mass in treatment C8 was lower than in C1 and C4 ($p<0.001$), in P8 lower than in P1 and P4 ($p<0.00001$). It was not different between C1 and P1 ($p=0.39$), neither between C8 and P8 ($p=0.16$). Such a pattern suggests that a high number of physical encounters between larvae decreases the body mass of resulting adults, regardless of pheromone amount, probably due to lower food intake following stress.

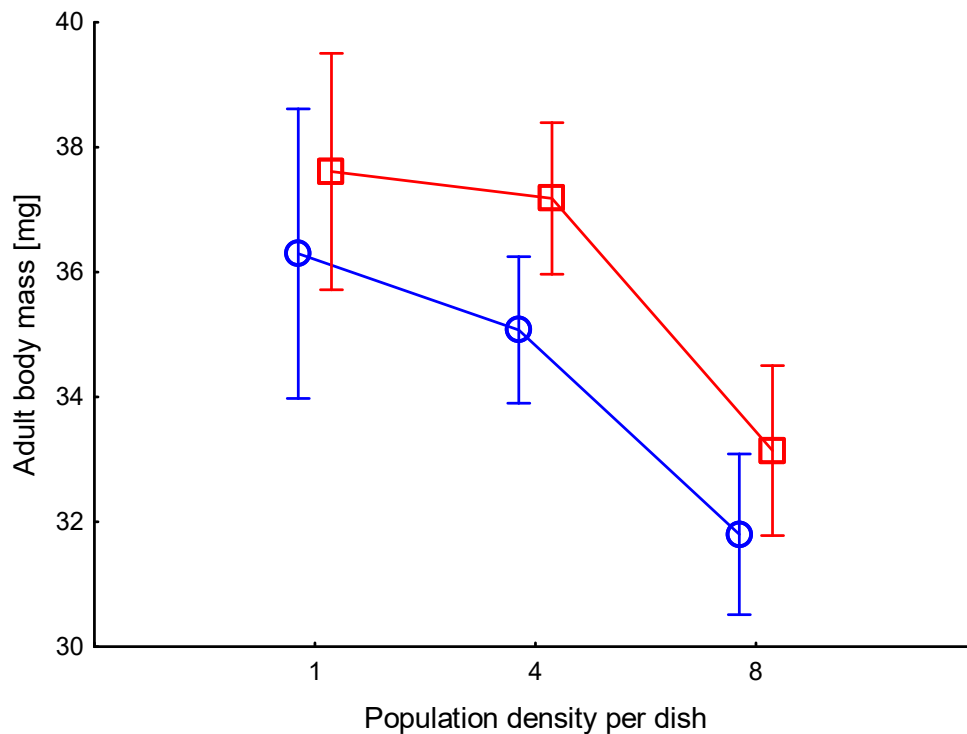


Figure 2. Body mass of one day old adults of *Harmonia axyridis* maintained as larvae in various numbers per 15 cm Petri dish and in Clean treatments (blue circles), and Pheromone treatments (red squares). Vertical bars denote 0,95 confidence intervals.

4. Discussion

4.1. Developmental time

When population density increased to the highest level, while the amount of putative pheromones was kept low (treatment C8), developmental time of the last instar larvae – the most voracious and cannibalistic – significantly increased. Unexpected frequent encounters with conspecifics seemed to delay metamorphosis to avoid becoming a defenseless pupal stage in the presence of many predators. Similar results were found in two Russian populations of *H. axyridis* [22], where an increase in population density (1, 5, and 10 per dish) led to an increase in development time. Other factors had a stronger effect than population density, with long photoperiod (18h) prolonging development, and diet consisting of aphid *Myzus persicae* accelerated development in comparison to *Ephestia* eggs diet.

A possible explanation for the differences in development times related to density and pheromones could be that high levels of stress from physical interactions and the presence of other individuals (competitors) may force individuals to compete for available resources (food, space, shelter). In this situation, individuals may prioritize short-term survival over development, taking more time to reach the pupal stage. This explanation was suggested for *Locusta migratoria* (Orthoptera: Acrididae) and *Aedes aegypti* (Diptera: Culicidae), where some of the effects of increased population density could produce immune responses and signs of nutritional deficiencies that affect developmental time [23,24].

The pupal development lasted 5.5 days in all treatments which corresponds to the values found in the literature, where the time spent in the pupal stage was 5.3 days at 24°C [25]. There was no delay either due to high density of larvae or pheromones deposited – pupae developed as fast as possible. They do not perceive the two types of information due to their limited sensory capacity, and they do not remember the situation from their previous life.

4.2. Adult body mass

Body mass of newly hatched adults, which is mostly dependent on mass gain during the last larval instar, also decreased only at the highest level of larval population density (treatments C8, P8). Similar results were found in the two Russian populations [26], where an increase in population density led to a significant decrease in body mass, but only in the treatment fed with aphids. Density-dependent factors, such as cannibalism and food availability, negatively affected larval development in both native and invasive Russian *H. axyridis* populations, leading to smaller adults. Because food supply was not limited in our experiments, and it consisted of *Ephestia* eggs, we consider the stress of frequent encountering the other larvae responsible to lower food ingestion or utilization.

Some authors also propose that competition for space indirectly affects the ability to feed and move efficiently. This trend was observed in flies *Musca domestica*, *Drosophila melanogaster*, the tropical butterfly *Bicyclus anynana*, and moth *Lymantria dispar* [27-31]. In all the studied cases, the population density was manipulated, finding that an increase in population density directly affects stress levels, producing a 10-45% decrease in the body mass of individuals, as well as other factors such as fertility and the effectiveness of sexual reproduction.

Ladybird females are always heavier than males [32], what was not distinguished in our samples. However, we found much higher average fresh mass than that reported for *H. axyridis* 28.3 ± 2.8 mg at long photoperiod females fed with *M. persicae* [33]. This difference suggests that food stress was not the direct driver of differences among our treatments.

However, the effects of density and pheromones were not positively combined. Perception of larval tracks (P treatments) increased the adult body mass, meaning that larval feeding and utilizing the food for growth was improved compared to clean environment (C treatments). Because each larva produces and then feels its own pheromones, it seems to be calmed from a possible stress in their presence. Known, already visited vegetation can be safer than unknown one.

4.3. General discussion

The trends found in this study caused by population density and deposited pheromones in both development time and body mass in *H. axyridis* warrants further investigation. Future studies should conduct a detailed analysis by increasing population density even further and observing changes in developmental time, body mass, and survival rate to determine a possible self-regulation mechanism of the species field populations at high densities. However, the effect of high larval density can be diminished in nature, where physical contact or encounters between individuals may cause them to take opposite paths and directions to avoid interaction and keep distance from each other.

Considering the scenario where an individual enters the pupal stage while surrounded by individuals in the larval stage, this individual would be defenseless and immobile, and could be cannibalized. Both the stress caused by the encounters leading to inefficient food intake and utilization, and endeavour to avoid defenceless stage are logical interpretations of our results. High-density populations may begin to self-regulate due to negative effects on growth and survival, reducing the risk of overpopulation. The presence of pheromones without physical contact with larvae could induce behaviors or physiological responses that accelerate development due to the activation of species regulation mechanisms.

5. Conclusions

Larvae of ladybirds seem to react both to their density and to the presence of larval tracks. High larval density had negative effects on developmental rate and subsequent adult body size. Presence of the tracks stabilized high developmental rate and increased body size. Thus, the compounds present in the tracks, previously known as oviposition deterring pheromone, can be also considered as development enhancing pheromones.

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