"Ontogenetic Changes in Feeding Behavior and Nutrient Allocation Patterns in Mexican beetle, *Zygogramma bicolorata* Pallister on *Parthenium hysterophorus* L."

Abstract

Parthenium hysterophorus L. (Asteraceae) is a noxious weed found in agricultural fields, pastures, and wastelands across tropical regions. It significantly reduces crop yields and poses serious health risks to humans. The Mexican beetle, *Zygogramma bicolorata* Pallister (Coleoptera: Chrysomelidae), has emerged as one of the most effective biological control agents against this weed. However, no previous research has examined the beetle's nutrient assimilation. This study evaluated the feeding behavior and nutrient assimilation in the larvae of *Z. bicolorata* when feeding on *P. hysterophorus*. The findings showed that the first instar larvae had the lowest food consumption rates and accumulated minimal amounts of glucose, proteins in their bodies. These larvae demonstrated compensatory feeding behavior, achieving the highest food utilization efficiency and developmental rates. In contrast, the fourth instar larvae exhibited the highest food consumption and conversion efficiency but had the slowest developmental rates. Notably, the fourth instar larvae accumulated the largest food reserves. These results provide valuable insights for laboratory-based mass-rearing programs for *Z. bicolorata*, though further field trials are necessary to validate these findings.

Keywords: *Ontogenetic changes, Feeding behaviour, Nutrient allocation, Zygogramma bicolorata, Parthenium hysterophoru*

Introduction:

Parthenium hysterophorus L. (Family: Asteraceae) is a highly invasive weed with a global distribution, predominantly affecting wastelands, agricultural fields, and pastures in Asia, Australia, and the American tropics, especially around the Gulf of Mexico (Navie et al., 1996). Biochemical studies have identified two key pseudoguaianolides, parthenin and coronopilin, within the plant, which suppress the growth of neighboring vegetation and cause hay fever, asthma, and allergic dermatitis in humans (McFadyen, 1995; De la Fuente et al., 1997; Das et al., 2007). Although chemical and mechanical control methods exist, they are often hazardous and unsustainable for long-term use. Biological control has emerged as the most cost-effective strategy, with the Mexican beetle, *Zygogramma bicolorata* Pallister (Coleoptera: Chrysomelidae), being one of the most effective biocontrol agents (Dhileepan et al., 2000; Cowie et al., 2018). Life stages of *Z. bicolorata*, particularly the fourth instars and adult females, are known for their voracious feeding habits, leading to extensive defoliation that promotes microbial activity on the leaves, reduces photosynthetic efficiency, and lowers seed production in *P. hysterophorus* (McConnachie, 2015; Hasan et al., 2018; Cowie et al., 2018). Adults lay eggs on various parts of the plant, including leaves, flower heads, stems, and buds, with emerging larvae initially feeding on inflorescences before moving to young leaves (Dhileepan et al., 2018). Pupation occurs in the soil, and the beetle's lifecycle spans 4–8 weeks, with up to four generations annually, depending on factors such as food availability, temperature, rainfall, and natural predators (Ray, 2011; Hasan and Ansari, 2016a, b; Chidawanyika et al., 2017). Adults have a lifespan of up to two years, spending approximately six months annually in soil during diapause (Dhileepan and Wilmot Senaratne, 2009). Nutritional requirements vary across the beetle's developmental stages, with larvae prioritizing growth and development, while adults focus on reproduction and oviposition

(Arrese and Soulages, 2010; Patel et al., 2020a). Biocontrol agents efficient in converting food into biomass tend to be equally effective in progeny production (Omkar et al., 2023). However, poor-quality diets or food scarcity can delay growth, development, and reproduction (Chidawanyika et al., 2017; Cowie et al., 2019; Patel et al., 2020a). Carbohydrates, primarily stored as glycogen, trehalose, and glucose, serve as key energy sources and support various physiological adaptations (Gaxiola et al., 2005; Kehl and Fischer, 2012). Zygogramma bicolorata, a leaf beetle native to North and Central America, was introduced to India in 1984 as a biological control agent against the invasive weed Parthenium hysterophorus (commonly known as Congress grass). In its native regions, the beetle is a natural herbivore of Parthenium. After its introduction to India, it successfully established itself and helped reduce Parthenium infestations, especially in southern regions. However, concerns arose about its non-target effects on native plants. Abroad, particularly in Mexico and the USA, it remains an indigenous species, playing a role in controlling native Parthenium. Its use demonstrates both the potential and risks of biological control agents.

Material and methods Stock maintenance:

Adult male and female *Zygogramma bicolorata* were collected from agricultural fields at Banaras Hindu University, Varanasi, India (25°20′N, 83°0′E), and differentiated based on the posterior margin of their last visible abdominal ventrite, which is intact in females but slightly serrated in males (Kumar, 2005). The adults were randomly paired in plastic Petri dishes (9.0 × 1.5 cm^2) and reared under controlled conditions (27 ± 2°C; 65 ± 5% RH; 14:10 L:D) in a Biological Oxygen Demand (BOD) incubator (NSW-152; Narang Scientific Works Pvt. Ltd., New Delhi, India) with fresh P. hysterophorus leaves provided daily. Eggs were collected every 24 hours, and first instar larvae were used for further experiments. The study consisted of two parts: the first evaluated feeding parameters (consumption rate, conversion efficiency, and growth rate) across the larval instars of Z. bicolorata under an unrestricted supply of P. hysterophorus leaves. The second measured concentrations of glucose, proteins, and triglycerides in larval instars after feeding. Newly hatched first instars (n = 100) were reared for two days on an ad libitum supply of P. hysterophorus leaves under the same abiotic conditions as the stock culture. On the third day, 50 larvae were weighed and placed individually in Petri dishes with pre-weighed P. hysterophorus twigs (approximately 500 mg biomass) for 24 hours under the same conditions. The larvae and leftover twigs were weighed after the experiment. A subset of larvae (n = 10) was analyzed for biochemical parameters, while the remaining (n = 40) were allowed to continue their development. This process was repeated for second (n = 40), third (n = 30), and fourth (n = 20) instars, identified based on head capsule width (~0.65 mm, ~0.98 mm, and ~1.44 mm, respectively). Biochemical estimations (glucose, proteins, and triglycerides) were conducted on 10 larvae from each stage, while the rest continued metamorphosis. Developmental durations for all larval stages were recorded, along with body size and biomass. To account for natural twig biomass loss, control twigs (n = 10; biomass = 500 mg) were kept under identical conditions, and the average loss was used to normalize data before calculating consumption rates, conversion efficiencies, and growth rates (modified from Patel et al., 2018).

Among the larval stages, third instars showed the highest food consumption and conversion rates at extreme temperatures (15°C and 30°C), while fourth instars achieved optimal conversion at 25°C, coupled with higher growth rates. At 30°C, third instars demonstrated superior growth rates compared to other stages. Regression analysis indicated that food consumption rates decreased with rising temperatures (15°C to 30°C), while conversion efficiencies and growth rates increased. First instars exhibited the lowest consumption, conversion, and growth rates, highlighting their relatively limited performance compared to later stages.

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Life cycle and feeding attributes

Freshly laid eggs from mating pairs of *Zygogramma bicolorata* were incubated at four constant temperatures: 15°C, 20°C, 25°C, and 30°C, with 100 eggs per temperature group. The eggs were placed in plastic Petri dishes (14.0 cm × 1.5 cm) and monitored daily to determine their incubation periods. Upon hatching, each first instar grub was weighed using an analytical balance (RA-200, Roy Electronics, India) and transferred to a separate Petri dish. The grubs were fed pre-weighed fresh twigs of *Parthenium hysterophorus* for 24 hours under the respective temperature conditions inside a BOD incubator. (Please provide the reference for the methodology aformentioned used) After this period, the grub was moved to a new Petri dish, and both the grub's biomass and the remaining twig biomass were recorded. This procedure was repeated for second, third, and fourth instar larvae under their designated temperature conditions. The developmental duration for each instar was measured across the different temperature regimes. Each experiment was replicated ten times to ensure reliability. The feeding parameters, including consumption rate, conversion efficiency, and growth rate of the grubs, were calculated at each temperature using established formulae (Patel et al., 2018).

Formula

$$\textbf{Consumption rate (mg/day)} = \frac{\text{Leaf } \textit{biomass consumed by the grub } (mg)}{\text{Feeding duration of the grub (days)}}$$

$$\textbf{Coversion efficiency} = \frac{\text{Increased } \textit{biomass of the grub } (mg)}{\text{Leaf biomass consumed by the grub (mg)}}$$

$$\textbf{Growth rate(per day)} = \frac{\text{Fresh mass gain of the grub (mg)}}{\text{[(Feeding duration (days)X(mean biomass of grub (mg))]}}$$

Estimation of glucose

Glucose quantification in the whole body homogenates of different instars of *Zygogramma bicolorata* was performed using a coupled colorimetric assay (Tennessen et al., 2014). Ten larvae from each developmental stage were homogenized in 1.5 ml centrifuge tubes containing 200 µl of cold phosphate-buffered saline (PBS). The homogenates were then centrifuged using a cooling centrifuge (REMI; NEYA 16R Refrigerated Centrifuge HIGH SPEED, 4x 175 ml, 16,000 rpm) at 12,000 rpm for two minutes. The supernatant from each sample was collected and placed in a water bath at 70°C for 10 minutes, followed by another centrifugation at 12,000 rpm for two minutes. The supernatant was transferred to fresh centrifuge tubes, and the samples were stored at −20°C in a mini cooler (TARSONS; 525030) for later analysis. For glucose standard preparation, a 1 mg/ml glucose standard was diluted with PBS to prepare a series of glucose standards at concentrations of 0.16 mg/ml, 0.08 mg/ml, 0.04 mg/ml, 0.02 mg/ml, and 0.01 mg/ml. Using a micropipette (transferpette® S digital 0.5–10 µl), 30 µl of the supernatant from each sample was transferred into wells of a flat-bottom 96-well microplate (TARSONS; 941196). Similarly, 30 µl of glucose standards and PBS were added to separate wells to serve as the standard and blank, respectively. To each well, 100 µl of glucose reagent (Sigma; GAHK20) was added. The microplate was sealed with ~~aluminum~~aluminium foil to prevent evaporation and incubated for 15 minutes for the reaction to occur room temperature.

Table 1: Estimation of Consumption rate, TAG, Proteins, and Glucose

Dependent variables	Covariate	Independent variables		
	Mean body biomass	Temperature	Stage	Interaction (Temperature ´ Stage)
Consumption rate (mg/ml)	F=0.001; P=0.985; df=1, 159	F=14.86; P<0.00010; df=3, 159	F=134.96; P<0.0002; df=3, 159	F=20.18; P<0.00010; df=9, 159
TAG (mg/ml)	F=2.04; P=0.154; df=1, 159	F=4.12; P=0.009; df=3, 159	F=9.07; P<0.00010; df= 3, 159	F=4.08; P<0.00010; df=9, 159
Proteins (mg/ml)	F=6.90; P=0.01; df=1, 159	F=5.24; P=0.003; df=3, 159	F=17.64; P<0.00010; df=3, 159	F=6.64; P<0.00010; df=9, 159
Glucose (mg/ml)	F=6.66; P=0.012; df=1, 159	F=7.36; P<0.00010; df=3, 159	F=14.74; P<0.00010; df=3, 159	F=5.25; P<0.00010; df=9, 159

Table 2: Two-way ANOVA

Two-way ANOVA Table showing the effect of mean body biomass (covariate), temperature, developmental stage and their interaction on consumption rate and basic body metabolites of *Zygogramma bicolorata* larvae (F-values significant at P<0.05)

Temperature	Stage	TAG (mg/ml)	Protein (mg/ml)	Glucose (mg/ml)
15°C	**First instar**	0.042 ± 0.001^{aC}	0.282 ± 0.007^{aB}	0.003 ± 0.000^{aA}
	Second instar	0.058 ± 0.002^{bB}	0.555 ± 0.016^{bA}	0.018 ± 0.0010^{bB}
	Third instar	0.688 ± 0.017^{cC}	3.039 ± 0.104^{cB}	0.109 ± 0.005^{cAB}
	Fourth instar	1.443 ± 0.056^{dC}	5.777 ± 0.202^{dB}	0.320 ± 0.035^{dB}
20°C	**First instar**	04 ± 0.0010^{aB}	0.274 ± 0.004^{aA}	0.004 ± 0.000^{aB}
	Second instar	0.057 ± 0.002^{bB}	0.515 ± 0.01^{bA}	05 ± 0.0010^{bC}
	Third instar	0.474 ± 0.031^{cB}	2.765 ± 0.067^{cAB}	0.126 ± 0.001^{cB}
	Fourth instar	1.416 ± 0.095^{dC}	5.292 ± 0.290^{dAB}	0.207 ± 0.004^{dA}
25°C	**First instar**	03 ± 0.0010^{aB}	0.287 ± 00^{aC}	0.003 ± 0.000^{aA}
	Second instar	0.089 ± 0.006^{bC}	1.190 ± 0.042^{bB}	02 ± 0.001^{bBC}
	Third instar	0.275 ± 0.034^{cA}	2.529 ± 0.271^{cAB}	0.107 ± 0.004^{cA}
	Fourth instar	0.934 ± 0.121^{dA}	5.211 ± 0.353^{dAB}	0.208 ± 0.036^{dA}
30°C	**First instar**	0.017 ± 0.001^{aA}	0.273 ± 0.004^{aA}	0.003 ± 0.000^{aA}
	Second instar	04 ± 0.002^{bA}	0.520 ± 0.013^{bA}	0.017 ± 0.0010^{bA}
	Third instar	0.344 ± 0.030^{cA}	2.404 ± 0.097^{cA}	0.095 ± 0.003^{cA}
	Fourth instar	1.188 ± 0.077^{dAB}	4.506 ± 0.249^{dA}	0.201 ± 0.006^{dA}

Basic body metabolite contents of larval stages of *Zygogramma bicolorata* under different temperature condition (Values are Mean ± SEM, small letters and large letters represent comparison of means amongst temperature conditions and amongst stages, respectively

Estimation of proteins

Protein quantification in the whole body homogenates of different larval instars of *Zygogramma bicolorata* was conducted using the Bradford protein assay (Bradford, 1976). The Bradford reagent was prepared using Coomassie Brilliant Blue G-250 and orthophosphoric acid. Ten larvae from each developmental stage were homogenized in a 1.5 ml centrifuge tube containing 200 µl of cold phosphate-buffered saline (PBS) using a homogenizer. The homogenates were then centrifuged at 12,000 rpm for two minutes. The supernatant from each sample was collected into fresh centrifuge tubes and stored immediately in a mini cooler at −20°C for further analysis. For protein standard preparation, Bovine Serum Albumin (BSA) protein standard (1 mg/ml) was serially diluted. To prepare sample solutions, 1 µl of the respective sample was mixed with 9 µl of distilled water in a centrifuge tube. A 10 µl aliquot of each sample solution was then transferred to a flat-bottom 96-well microplate. The serially diluted BSA standards were also added to the microplate, and distilled water was transferred into two wells to serve as blanks. To each well, 200 µl of Bradford reagent was added, and the contents were mixed well. The microplates were sealed with ~~aluminum~~ aluminium foil to prevent evaporation and incubated for 8 minutes at 25°C. The total absorbance was measured at 595 nm using a microplate reader. Standard curves were created by plotting the net absorbance against the protein concentration of each BSA standard, and the protein concentration in the samples was determined by comparing their absorbance to the standard curves.

Statistical analysis:

The dependent factors, including feeding attributes (such as consumption rate, conversion efficiency, and growth rate), mean body biomass, developmental rates, and concentrations of glucose, proteins, and triglycerides in the haemolymph, were analyzed using one-way ANOVA. Tukey's post hoc comparison of means was applied, with larval instars as the independent factor in the analysis. All statistical analyses were performed using MINITAB 16 (Minitab Inc., Pennsylvania, USA).

Results and discussion:

The feeding attributes, nutrient assimilation, and mean body biomass of *Zygogramma bicolorata* larvae varied significantly across larval stages (Tables 1 and 2). Analysis of the data revealed that as the larvae progressed through subsequent stages, both their consumption rates and mean body biomass decreased. The fourth instar larvae were the heaviest and exhibited the highest consumption rates compared to the other instars. They also accumulated the maximum concentrations of glucose, proteins. In contrast, the first instar larvae were the lightest and had the lowest consumption rates, with significantly lower nutrient accumulation in their bodies compared to the later instars (Table 2). The higher consumption rates in the fourth instars are likely due to their larger size (8.00 ± 0.22 mm) compared to the smaller first (1.00 ± 0.02 mm), second (2.27 ± 0.06 mm), and third (3.22 ± 0.06 mm) instars. Larger size is associated with increased metabolic costs (Omkar and Afaq, 2011; Patel et al., 2020a), so the fourth instars likely consume more weed biomass to meet their higher energy demands and complete their development. Conversely, the smaller size and lower energy requirements of the first instar larvae likely explain their reduced consumption rates and body biomass, leading to lower nutrient assimilation. These findings are consistent with previous studies on coccinellid beetles (Mishra et al., 2012; Kumar et al., 2014; Wu et al., 2018). Additionally, significant differences were observed in the growth rates, conversion efficiencies, and developmental rates of the larval instars (Table 1). The higher growth rates of the first instars may be attributed to their small size (Patel et al., 2020b), and despite their lower consumption rates, their higher food utilization efficiencies suggest that they compensate for reduced food intake by accelerating their growth. This compensatory feeding

likely enables first instar larvae to efficiently exploit available nutrients and accumulate enough energy reserves for the next stage of development. Similar compensatory feeding patterns have been documented in coccinellid beetles (Mishra et al., 2012).

Conclusion:

Thermal stress has a considerable impact on the growth, development, food utilization, and nutrient mobilization of *Zygogramma bicolorata*. Increased temperatures negatively affect the larval and pupal development stages, resulting in longer life cycles and reduced growth efficiency. Under thermal stress, food utilization efficiency—including ingestion, digestion, and conversion rates—decreases, leading to lower biomass accumulation and depleted energy reserves. Additionally, the beetle's ability to mobilize essential nutrients such as proteins, lipids, and carbohydrates, which are vital for growth and reproduction, is hindered. These physiological disruptions undermine the beetle's survival and its potential as an effective biological control agent for invasive weeds like *Parthenium hysterophorus*. Understanding how varying thermal conditions affect these processes is essential for assessing *Z. bicolorata's* adaptability to climate change and its continued success in biological control programs. Future research should focus on strategies to improve thermal tolerance and preserve the beetle's ecological function amidst global warming challenges.

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