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Behavioural Responses of *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae) to *Paracoccus marginatus* (Papaya Mealybug) Related Cues in Laboratory Conditions

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ABSTRACT

Purpose: *Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) is a well-known biocontrol agent that is widely used to control soft-bodied insects including papaya mealybug. This study aimed to evaluate the behaviour of C. montrouzieri to chemical simulants associated with honeydew (HD) and crushed papaya mealybugs (CPMBs), under laboratory conditions.*

Research Method: *The larvae and adult stages of C. montrouzieri were introduced into a Petri dish containing HD or CPMBs treated and untreated Carica papaya (Papaya) leaf discs, and the behaviour was recorded for 20 minutes using video surveillance. Searching time, walking speed, and number of turns on leaf discs were quantified.*

Findings: *Results revealed that the searching time of C. montrouzieri, on papaya leaf discs was significantly varied with different larval instars and adults (F $_{(4,298)}$ = 17.21, p<0.05) as well as leaf-coated substances (F $_{(2,298)}$ = 78.07, p<0.05). When considering all larval instars of C. montrouzieri searching time on CPMBs (690.7 ± 25.3 s) was significantly higher compared to HD (524.8 ± 33.5 s) and clean leaf disc (219.6 ± 34.4 s) (F $_{(2, 227)}$ = 60.61, p<0.05). The searching period of the first larva (L1) was significantly higher compared to adults in all treatments (p<0.05). The walking speed of the fourth larva (L4) and adults of C. montrouzieri on HD and CPMBs leaf discs was significantly lower compared to that of clean leaf discs. The number of turns per unit path length was significantly higher in both HD (1.100 /cm) and CPMBs (1.129 / cm) leaf discs compared to the control (0.501 /cm) concerning larvae and adults.*

Originality/value: *It leads to the conclusion that HD and CPMBs considerably change the searching behaviour of larvae and adults of C. montrouzieri. Further work should be focused on examining and utilizing these chemical cues for searching to enhance the efficacy of C. montrouzieri in controlling papaya mealybug.*

Keywords: *Crushed mealybugs, Cryptolaemus, honeydew, Paracoccus marginatus, searching behaviour, walking*

INTRODUCTION

Paracoccus marginatus Williams Granara de Willink, better known as the Papaya Mealybug (PMB), is a soft-bodied insect belonging to the family Pseudococcidae (Hemiptera; Sternorrhyncha), poses a substantial threat to agriculture due to its ability to feed on over 200 host plants, including the fruit crop, papaya

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(*Carica papaya* L.) (Finch *et al*., 2020). PMB as well as other Homopteran insects (*i.e.*, aphids, whiteflies, scale insects, etc.) excrete honeydew which contains sugar, amino acids, and waxes (Leroy *et al*., 2014). These substances are directly derived from phloem sap and are sometimes synthesized by the insect itself (Buitenhuis *et al*., 2004). Honeydew acts as a source of carbohydrates for its foragers, (*i.e.*, ants, predators, and parasitoids) (Buitenhuis *et al*., 2004). Honeydew also acts as an info-chemical for foraging parasitoids and predators (Buitenhuis *et al*., 2004). The chemicals associated with honeydew are often described as 'kairomones' (Heidari and Copland, 1993). Kairomones are highly volatile compounds by nature and attract foragers or facilitate them for intensive searches in the host locations and subsequently, increase the chance of prey encounter (Heidari and Copland, 1993). Mealybug-infested leaves often get contaminated with wax and possibly with honeydew (Meiracker *et al.*, 1990).

Many studies revealed that honeydew and wax attract adult-stage predators and parasitoids and honeydew and/or wax act as an arresting stimulus (Leroy et al., 2014). An arrestant is a chemical substance that causes insect aggregation by indirect kinetic reactions, either by slowing down or stopping the movement (Leroy *et al*., 2014). Waage (1978) has categorized the insect arrestment responses into several categories namely: orthokinetic response (dent the walking speed) or boost turning which can be non-directional (klinokinetic response) or unidirectional (klinotactic response). The searching behaviour of predators and parasitoids has been studied by several authors using different methods and developed some mathematical models that can be used to describe the outcome of a predator or parasitoid search (Evans, 1976).

Olfactory cues accelerate the chance of prey encounters and thereby enhance the efficacy of predators or parasitoids (Purandare and Tenhumberg, 2012). Location of prey by immature life stages of predators (*i.e.*, larva), occurs without involving visual cues (Carter and Dixon, 1984). However, coccinellids show increased searching for a location, followed by catching each prey (Carter and Dixon, 1984). Most of the previous studies regarding honeydew and the searching behaviour of foragers have been done for parasitic hymenopterans and few studies have focused on predatory coccinellids (Carter and Dixon, 1984; Purandare and Tenhumberg, 2012; Leroy *et al.*, 2014).

Starvation is an internal factor that alters the host-searching behaviour of predators (Sengonca *et al.*, 1995). Prolonged starvation leads to intensive prey searching and directional movement towards the host (Grettenberger and Joseph, 2019). Gui and Boiteau (2010) reported that starvation is a trigger of insect dispersal. The starvation level may mediate the searching behaviour of foragers; for instance, predators alter their search path with starvation (Maselou and Fantinou, 2016). In contrast, some authors reported that there is no positive correlation between walking distance or speed, and starvation (Wallin and Ekbom, 1988; Hénaut *et al.*, 2002Pyralidae; Bengtsson *et al.*, 2004 Lamine *et al.*, 2005).

Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) is a key biocontrol agent that is widely used to control soft-bodied insects including papaya mealybug (PMB) (Paraiso and Services, 2016). Adult *C. montrouzieri* utilizes visual and chemical cues to locate its host (Heidari and Copland, 1992; Heidari and Copland, 1993). Further, these cues enable predators to gather information regarding the density and quality of host insects in a specific habitat (Jayanthi *et al.*, 2012). Some researchers argued that the larvae of this coccinellid perceive prey only by physical contact (Heidari and Copland, 1992). It is, however, evident that mealybug secretions such as wax and honeydew act as arresting and oviposition stimulants for *C. montrouzieri* (Heidari and Copland, 1993; Merlin *et al.*, 1996). In previous research, few studies have focused on the ability of honeydew (kairomones) to mediate host finding in *C. montrouzierei*. The primary objectives of this study were to examine the behaviour of all predatory stages of *C. montrouzieri* (L1 to adult) in response to honeydew and chemical substances associated

with whole-body crushed papaya mealybugs. Furthermore, this study compares the results with previous findings to identify any discrepancies and suggest areas for future investigation.

MATERIALS AND METHODS

Insect Cultures

PMB and *C. montrouzieri* adults were collected by field sampling of infested papaya plants. Colonies of mealybugs and predators were maintained under laboratory conditions at 26 ± 2 °C, 65-85% relative humidity. *C. montrouzieri* was reared on *Pseudococcus viburni* (Pseudococcidae) infested fruits of *Cucurbita moschata* (pumpkin) according to the method described by Gunawardana and Hemachandra (2020). The ovisacs of PMB required for the experiments were obtained from cultures maintained on potato sprouts (*Solanum tuberosum* L.) following the method described by Nisha and Kennedy (2017). *C. montrouzieri* has four larval instars, and those were identified using the morphometric description by Özgökçe *et al.,*(2006). All larval stages and adults used in these tests were uniform in size and belonged to the same generation.

Honeydew Collection and Leaf Disc Preparation

Honeydew was collected by placing Petri dish lids under potato sprouts that were heavily infested with *P. marginatus*, for 2-3 days. One honeydew droplet was carefully taken using a camel hair paintbrush and diluted with distilled water (1:1 v/v) applied on the abaxial surface of the prepared papaya leaf disc and allowed to air dry.

Extraction of Mealybugs

Three mature adult mealybugs were crushed with a pin and releasing body fluid was diluted with distilled water (1:1 v/v) applied on leaf discs as above and allowed to air dry completely. Distilled water was applied in the control treatment.

Location Behaviour of the Predator, C. montrouzieri

Circular leaf discs of *Carica papaya* (Papaya) without main veins (30 mm in diameter) were placed upside down and glued in Petri-dishes (9cm, in diameter). The papaya leaf discs were treated separately with PMB honeydew crushed mealybugs and glued in the centre of the Petri dish before the test. The treated leaf discs were then offered to larvae (*viz.,* L1 – L4) and adults of *C. montrouzieri*, and their activities were recorded by a digital camera (Full HD) for 20 minutes (Fig. 01.). The predator larvae were released into the experimental arena using a soft camel-hair paintbrush. The larvae and adults utilized in the experiments were obtained from rearing units, which were carefully maintained to ensure consistency in the physiological age of the coccinellids and subjected to a shorter starvation period before the test (6h). Each treatment was evaluated for each larval stage and adult with 18-24 replicates.

In all the experiments, Petri dishes containing leaf discs were placed above a fluorescent light and under a high-resolution video camera. Tests were carried out between 8:00 and 16:00 hours, to avoid any diurnal variation. The treatment order was randomized on each tested day. At the start of each experiment, one individual was placed inside the lid of the Petri dish. The individuals who were not coming into contact with the edge of a leaf disc within the video recording time were discarded. Termination of one visit was decided when the predators walked off the treated leaf disc for more than 3 minutes when it flew off from the arena or when it reached the sides or the top of the Petri dish. The arena was cleaned after each run with clean water successively and allowed to air dry after each replicate and all leaf discs were used only once. The movements of the predatory stage inside the Petri dish on

papaya leaf discs were traced onto a transparent sheet and photographed. Total path length and net travel distance in tracing photographs were measured using '*ImageJ*' software. Then, the traced search paths were used to calculate the walking speed (mm/s). Turning ($>90^0$) per unit distance travelled (1 cm) was calculated for all the treatments. (Eq-01).

Turns per cm= No. of turns (>90 degree)/Path Length   *Eq01*

Data Analysis

The data relating to different behavioural parameters were analysed in one-way and two-way analysis of variance (ANOVA) and means were separated using Tukey's HSD multiple range test at $p<0.05$. All statistical tests were conducted using Minitab software (version 19, Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

The searching time of *C. montrouzieri*, on papaya (*Carica papaya* L.) leaf discs was significantly varied with different larval instars and adults ($F_{(4,298)} = 17.21$, $p<0.05$) as well as leaf-coated substances ($F_{(2,298)} = 78.07$, $p<0.05$). The interaction between leaf coat substances and actively feeding stages; larval instars and adults, was also found significant ($F_{(8,298)} = 2.74$, $p = 0.006$).
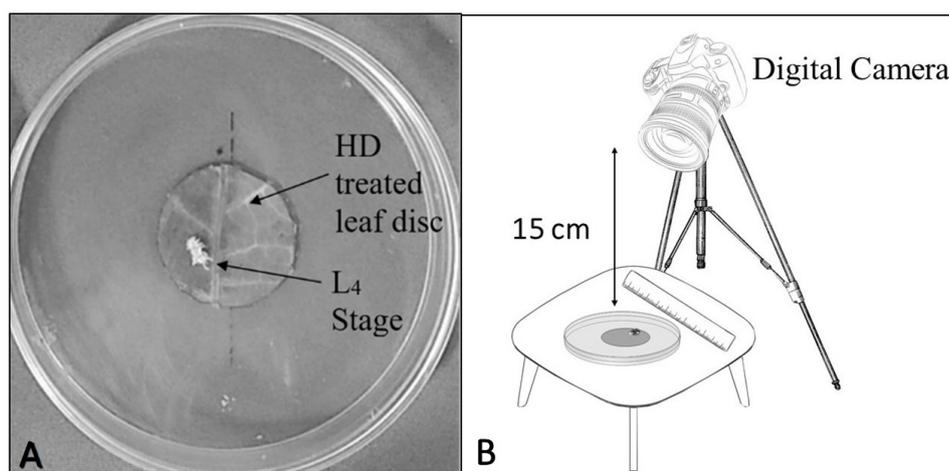

Figure 01:   **L4 larvae exposed to honeydew treated leaf disc (A) and video recording set-up with fixed camera (B).**

Table 01:   **The searching period of different stages of *C. montrouzieri* on honeydew, crushed papaya mealybug, and clean leaf discs.**

Larval instar/ life stage	Searching time on leaf disc (s)		
	HD	CPMBs	Clean (control)
I	674.3 ± 71.0^{aA}	747.8 ± 51.4^{aA}	270.1 ± 71.0^{bA}
II	536.6 ± 48.1^{aAB}	613.5 ± 42.3^{aA}	195.0 ± 71.0^{bA}
III	494.3 ± 63.3^{bAB}	714.8 ± 46.2^{aA}	222.1 ± 60.8^{cA}
IV	393.8 ± 57.1^{bAB}	686.9 ± 43.7^{aA}	191.1 ± 58.9^{cA}
Adult	374.7 ± 44.5^{aB}	278.8 ± 38.2^{aB}	45.7 ± 57.1^{bB}

When the larval instars were on honeydew-coated leaf disc, searching time was not significantly different among larval instars and the average searching time was 524.8 ± 33.5 s. Moreover, the searching time of larval instars on CPMBs leaf coats was also not significantly different; the average searching time of larval instars on CPMBs was 690.7 ± 25.3 s. The average searching time on clean leaf discs was 219.6 ± 34.4 s. The searching time of adults was significantly different to all larval instars when on both in CPMBs ($F_{(4, 140)}$ = 21.88, $p<0.05$) and clean leaf coat treatments ($F_{(4, 70)}$ = 8.00, $p<0.05$) (Table 01).

When the first larval instar (L1) was considered across the treatments: honeydew, CPMBs, and clean leaf discs, the searching time was significantly higher both in honeydew and CPMBs leaf coats compared to clean leaf disc ($F_{(2, 40)}$ = 10.78, $p<0.05$). Similar observation was observed with second (L2) ($F_{(2, 68)}$ = 12.55, $p<0.05$), third (L3) ($F_{(2, 51)}$ = 18.26, $p<0.05$) and fourth (L4) larval instars ($F_{(2, 59)}$ = 26.33, $p<0.05$). The searching times of L3 and L4 instars on CPMBs coated leaf disc were significantly higher compared to both HD and clean leaf discs (Table 01). Further, the searching period of L1 was significantly higher compared to adults in all treatments ($p<0.05$). This behavioural difference may enable first instar larvae to locate

prey immediately which is essential for their survival (Leroy *et al.*, 2014). Additionally, when considering all larval instars of *C. montrouzieri* searching time on CPMBs (690.7 ± 25.3 s) was significantly higher compared to HD (524.8 ± 33.5 s) and clean leaf disc (219.6 ± 34.4 s) ($F_{(2, 227)}$ = 60.61, $p<0.05$) (Fig. 02.).

These findings strongly suggest that HD and CPMBs coatings contain some compounds which may be utilised as chemo stimuli by larval and adult stages of C. montrouzieri. Further, searching more intensively in areas that contain HD or CPMBs, increases the chance of locating prey (klinokinatic response). Previous studies have shown that adult C. montrouzieri was significantly attracted to mealybug sex pheromones and showed a high searching period when mealybugs were in the vicinity (Heidari and Copland, 1992). Honeydew and other substances associated with the host mealybugs facilitate sustaining the predator in the contaminated areas. Heidari and Copland (1993) observed that the adult C. montrouzieri spent 98 % of their observed time (viz., 300 s) on the honeydew-treated leaf, whereas it spent 45 % on clean leaves. Purandare and Tenhumberg (2012) found that Hippodamia convergens Guérin-Méneville (Coleoptera: Coccinellidae) larvae spend a longer period in honeydew-coated patches than in clean patches after contact.
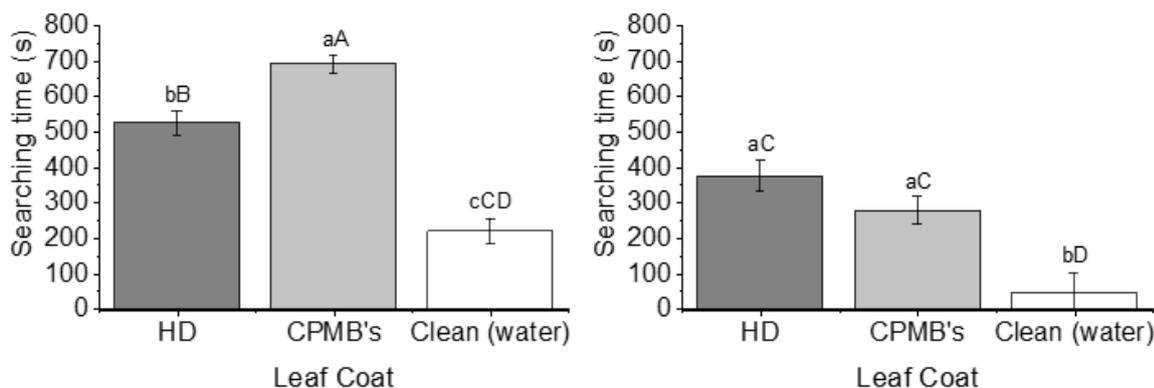

Figure 02: **Searching period of *C. montrouzieri* larval stages (left) and adults (right) on papaya leaf discs. (All bars indicate SE. Means followed by the same lowercase letter within the same graph are not significantly different (*p>0.05*). Means followed by the same uppercase letter between two graphs are not significantly different (*p>0.05*) by Tukey's HSD test)**

Resting time (on leaf disc) was significantly varied with predatory life stage ($F_{(4, 298)}$ = 9.19, p<0.05), but not significantly varied with leaf-coated material. Young instars of *C. montrouzieri* (L1, L2, and L3) showed a significantly higher resting time in all treatments whereas L4 and adults showed a significantly lower resting period (Fig. 03.). Similar observations for adults were reported by (Heidari and Copland, 1993), where the resting time was not significantly different between honeydew treated and clean leaves. In contrast, the same authors reported that the resting period of L4 significantly differed between honeydew-treated leaves and clean leaves. These differences probably resulted due to differences in host mealybug, leaf material, and maturity of L4 larvae.

Walking speed was significantly varied concerning different larval instars and adults (F (4, 736) = 647.3, *p*<0.05) and leaf-coated substances (F $_{(2, 736)}$ = 121.9, *p*<0.05) in all treatment combinations. The interaction between leaf-coated substances and the predatory life stage also significantly affected *C. montrouzieri* walking speed on papaya leaf discs (F $_{(8, 736)}$ = 75.7, *p*<0.05). Adults of *C. montrouzieri* showed the highest walking speed in all treatments while the first larval instar (L1) showed the lowest walking speed in all treatments. Moreover, there was no significant difference in walking speed between L1 and L2 as well as between L3 and L4 larval instars in all treatments (Table 02).

The walking speed of L4 and adults of *C. montrouzieri* on HD and CPMBs leaf discs was significantly lower compared to that of control/ clean leaf discs. Further, larval instars and adults of *C. montrouzieri* walk significantly faster outside the leaf disc in HD and CPMBs coated papaya leaf discs (*p*<0.05), but significant difference was not observed in and outside the leaf disc for L2, L3, and L4 in control/ water coated leaf discs. Moreover, adults of *C. montrouzieri* were walking faster outside the papaya leaf disc (12.35 ± 0.14 mm/s) compared to larval stages. Adults showed the highest walking speed on clean leaf discs (9.26 ± 0.21 mm/s) and it was significantly reduced to 3.98 ± 0.12 mm/s on HD-coated leaf

discs and 3.68 ± 0.13 mm/s on CPMBs coated leaf disc. These findings are in accordance with Banks (1957) who reported that *Propylea quatuordecimpunctata* (L.) (Coleoptera: Coccinellidae) larvae moved slowly on honeydew-coated bean leaves compared to clean bean leaves (orthokinesis) and increased turnings (klinokinesis). The walking speed of early instars (*viz*., L1, L2 and, L3) was not significantly varied concerning different leaf-coated substances (*p*>0.05), which was not observed in L4 and adults. Wratten (1973)Adalia bipunctata (L. also reported that larger instars of *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) move more rapidly than small ones. It was noted that the walking speed of the fourth instar larvae of *C. montrouzieri* is somewhat low (2.41 mm/s) in the honeydew-treated area compared to the value reported by Heidari and Copland (1993) (*i.e.,* 4.81 mm/s). This is probably due to the maturity level of larvae (middle age fourth instar larvae). Heidari and Copland (1993) used fourth young instar larvae – that were just after moult – which move faster. Moreover, several internal and external factors determine insect movements, such as temperature, relative humidity, airflow, host density, level of starvation, the intensity of light, prey secretions, and host-bearing plants (Heidari and Copland, 1992). Heidari and Copland (1993) argued that the availability of honeydew is caused by intense searching by both adults and larvae of *C. montrouzieri* with an increased number of turns and reduced walking speed.

The number of turns (>90^0) for 1 cm of the walking path is given in Table 03. The number of turns per 1 cm of the walking path was significantly varied with leaf-coated substances ($F_{(2, 457)}$ = 58.5, *p*<0.05), but not with predatory life stages. The number of turns per unit path length was significantly higher in both HD (1.100 /cm) and CPMBs (1.129 /cm) coated papaya leaf discs compared to clean/control leaf discs (0.501 /cm) concerning all *C. montrouzieri* stages (klinokinesis).
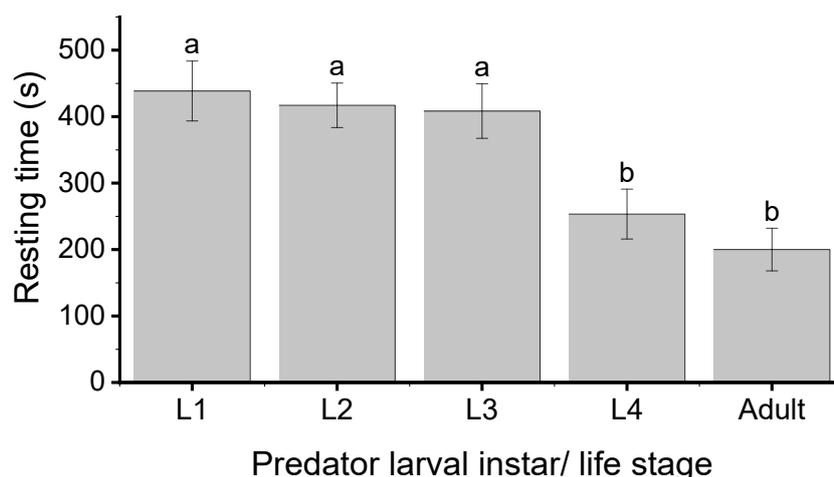
Figure 03: Resting time of *C. montrouzieri* on papaya leaf discs. (All bars indicate SE. mean values capped by the same letter are not significantly different (Tukey's HSD test; *p>0.05*)).

Table 02: Walking speed (mm/s) of *C. montrouzieri* inside and outside the leaf disc in different treatments.

Treatment	Larval instar/ life stage	(a) Walking speed Inside the treated zone (mm/s)	(b) Walking speed outside the treated zone (mm/s)
HD	I	0.55 ± 0.23^{fg}	$1.67 \pm 0.67^{*}$
	II	0.91 ± 0.11^{fg}	$1.85 \pm 0.35^{*}$
	III	2.31 ± 0.19^{de}	$3.39 \pm 0.32^{*}$
	IV	2.41 ± 0.18^{de}	$3.58 \pm 0.42^{*}$
	Adult	3.98 ± 0.08^{b}	$11.67 \pm 0.18^{*}$
CPMBs	I	0.56 ± 0.13^{g}	$1.63 \pm 0.42^{*}$
	II	1.11 ± 0.09^{fg}	$1.97 \pm 0.31^{*}$
	III	2.24 ± 0.21^{de}	$3.08 \pm 0.25^{*}$
	IV	2.39 ± 0.15^{de}	$3.24 \pm 0.42^{*}$
	Adult	3.68 ± 0.09^{bc}	$12.35 \pm 0.19^{*}$
Control (Water)	I	0.69 ± 0.20^{fg}	$1.63 \pm 0.67^{*}$
	II	1.57 ± 0.20^{ef}	1.82 ± 0.49^{ns}
	III	2.91 ± 0.23^{cd}	3.03 ± 0.47^{ns}
	IV	3.65 ± 0.20^{bc}	3.32 ± 0.43^{ns}
	Adult	9.26 ± 0.14^{a}	$12.12 \pm 0.21^{*}$

Notes- HD, and CPMBs indicate honeydew and crushed papaya mealybug-coated leaf discs, respectively. Different letters in column (a) have significant differences. ^{ns} and * in column (b) indicate no significant and significant differences within the same raw at p<0.05 by Tukey's HSD test.

Table 03: **Number of turns (>90^0) for 1 cm of walking path of *C. montrouzieri* under leaf coatings of HD and CPMBs.**

Treatment	Larval instar/ life stage	Number of turns (>90^0) for 1 cm of the walking path
HD	I	1.24 ± 0.10^a
	II	1.06 ± 0.05^{ab}
	III	0.98 ± 0.07^{ab}
	IV	1.13 ± 0.12^{ab}
	Adult	1.08 ± 0.11^{ab}
CPMBs	I	1.16 ± 0.07^a
	II	1.12 ± 0.05^a
	III	1.13 ± 0.05^a
	IV	1.16 ± 0.05^a
	Adult	1.05 ± 0.06^{ab}
Control (Water)	I	0.55 ± 0.09^c
	II	0.36 ± 0.16^c
	III	0.44 ± 0.19^{bc}
	IV	0.46 ± 0.11^c
	Adult	0.67 ± 0.12^{bc}

Notes- HD, and CPMBs indicate honeydew and crushed papaya mealybug coated leaf discs, respectively. Different letters indicate no significant and significant differences within the column at p<0.05 by Tukey's HSD test.

However, since 1980, host searching behaviour of coccinellid was considered random and do not perceive their prey until contact, which has been challenged by several authors (Carter and Dixon, 1984; Sengonca, *et al.,* 1995; Purandare and Tenhumberg, 2012; Urbina *et al.*, 2018; Chun-li *et al.*, 2019). The length of time a coccinellid spends searching for food is not the same for all individuals of the same developmental stage (instar). Instead, it may vary and be influenced by the hunger level of the individual coccinellid. In other words, a hungry ladybird beetle may search for food more intensively and for a longer period than a well-fed one. (Bond, 1980; Carter and Dixon, 1982). Further, Sengonca *et al.*, (1995) reported that the maximum olfactory response was observed in the first to fourth instar larvae and adults of *C. montrouzieri* after a period of starvation lasting 4, 8, 12, 12, and 24 hours, respectively. Previous studies have shown that predatory coccinellid larvae use honeydew as a kairomone while searching for host aphids. Stubbs (1980) found that adults of a seven-spotted ladybird, *Coccinella septempunctata* (Coleoptera: Coccinellidae) can identify host aphids at a short distance by visual cues whereas their larvae move towards crushed prey at a short distance which was identified as an olfactory detection. Stubbs (1980) and Carter and Dixon, (1984) showed *C. septempunctata* larvae stay a longer period on plants grown on honeydew-contaminated soil compared to the control and found that the arrestment response of this coccinellid larva was elicited by the chemicals associated with the haemolymph of crushed prey. Carter and Dixon (1984) found *C. septempunctata* larvae increase their searching time in ears of wheat that carry honeydew than the control. Further, Pettersson *et al.* (2008) showed that chemical stimuli are important in the foraging behaviour of *C. septempunctata* L.

Merlin *et al.,* (1996) found that the wax secretions and honeydew of host mealybugs were used as an attractant and oviposition stimulants by *C. montrouzieri*. An olfactory study done by Sengonca *et al.* (1995) pointed out that the first instar larvae and adults of *C. montrouzieri* were significantly attracted to the kairomones of *Planococcus citrl* (Risso). They also revealed that all the stages of *C. montrouzieri* were attracted to kairomones produced by mealybug *P. citri* significantly (*p<0.05*) indicating *C. montrouzieri* uses the kairomones of mealybug as cues in reaching the prey.

CONCLUSIONS

The study revealed that the searching time of *C. montrouzieri*, on papaya leaf discs significantly varies with different feeding stages of the predator, and also with leaf-coated substances. Further, all feeding stages of *C. montrouzieri* spend more time searching in leaf discs coated with honeydew and CPMBs compared to clean leaf discs (klinokinatic response). Hence, honeydew, and whole-body crushed papaya mealybug serve as behavioural stimulants of *C. montrouzieri*, under laboratory conditions. Further work on the examination or synthesis of these compounds may enhance the efficient use of *C. montrouzieri* for population suppression of mealybugs.

ACKNOWLEDGEMENT

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