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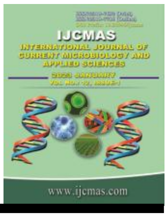

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Pupal Dimorphic Coloration is Sex-Specific in *Harmonia axyridis* - Kuwait

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ABSTRACT

Keywords

Pupae, laboratory, ladybird, temperature, sex, adult, larvae, pigmentation, melanin

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This study is the first to use pupal pigmentation of the ladybug *H. axyridis*, a species from Kuwait, to identify sex prior to adult emergence. Twenty adults were collected from fields and reared under laboratory-controlled measures. Based on preliminary observations, it was hypothesized that orange pupae would develop to females, whereas males would emerge from dark pupae. 100 orange & 100 dark brown were collected from the second generation of the reared colony and each reared under similar laboratory conditions. Ninety-seven orange pupae developed into females, whereas 94 dark brown pupae developed into males. Another two hundred pupae were collected randomly from the field, each in a separate Petri-dish, and were left to grow in the field. Ninety-six percent of orange pupae developed into females, whereas ninety-nine percent of males developed dark brown pupae. For validation, the mean total area of melanin pigmentation present on the dorsal surface of pupae was measured using Image-J software and the results revealed significant variations for both field and laboratory samples of different colors. The overall outcome was consistent with the proposition that the pupal dimorphic colorsare sex-specific and could be applied to identify the sex prior to adult emergences in *H. axyridis*.

Introduction

The ladybug *Harmonia axyridis* (Coleoptera: Coccinellidae) is commonly known as the harlequin, multicolored Asian beetle (Koch *et al.*, 2006). Despite the fact it is an effective biological control agent in agriculture, it became a threat posing a noteworthy risk, particularly to aphids' community, indigenous species and guild biodiversity (Majerus *et al.*, 2006; Bahlai *et al.*, 2015). This species is broadly considered the most invasive and nuisance insect worldwide (Vilcinskas, 2013). It is able to thrive across a wide range of climatic conditions with a voracious appetite enabling it to become a strong competitor among other ladybirds.

The easiest way to identify the sex of *H. axyridis* is from the secondary reproductive structures as described by Majerus (1994). In 2007, McCornack and his team reported that the distal region of the 5th abdominal sternites could be used to find the sex ofharlequin adults however, this often entails microscopic aids and might be subjected to individual decisions.

Galvan *et al.*, (2008) suggested a handier protocol for distinguishing the sex of *H. axyridis* based on the degree of pigmentation on the labrum and prosternum that looks darker in males than females. Yet, all the existing means could disrupt the insect and are not easy to apply, thus, a new reliably accessible method is required for sex identification.

Preliminary laboratory investigations showed that there was a significant dissimilarity in pupal pigmentation of *H. axyridis* present in Kuwait. It was noted that some of the pupae appeared either dark brown or orange morphs. Most of the dark pupae gave rise to males, while the females emerged from orange ones. Pupae of insects had known to have a wide range of coloration inter- and intra-species.

For example, Yamanaka *et al.*, (2006) reported the phenotypic plasticity in pupal pigmentation in the swallowtail butterfly, *Papilio Xuthus*. Hiraga (2005) also noticed the appearance of dual pupal coloratio in thebutterflies *Graphium sarpedon nipponum*.

Several factors have been recognized to control pupal color polymorphism; such as light quality and intensity, ambient temperature, relative humidity, nutrient availability, hormonal influences, time of larvae pupation, the strength of sex gene expression, and the sensitivity to the substrate characteristics (Hiraga, 2006; Nunney, 2007; Yamanaka *et al.*, 2009; Yamamoto, 2011).

The multicolored Asian ladybird *Harmonia axyridis* exhibits a wide variety of cuticular color in the form of melanin spot patterns and quantity on elytra. Nevertheless, it was confirmed that the pupae of *H. axyridis* could be the critical stage determining the final elytra pigmentation. Yet, no study has explored the underlying reasons for having the different pupal coloration in *H. axyrids* and do these colors differ between gender. Based on a preliminary laboratory observation by this there was a significant dissimilarity in pupal pigmentation of *H. axyridis* found in Kuwait (Fig.1).

It was noted that some of the pupae appeared dark brown and others light morphs. Most of the dark pupae gave rise to males, while the females emerged from light ones.

Materials and Methods

To establish the colony, twenty adults of the ladybeetle *H. axyridis* were collected randomly from Al-wafra farm in Kuwait in March 2020. Two hundred out of three hundred pupae collected from the second generation were separated into two groups orange (O1) and black pupae (B1) and each pupa was kept in a 9 cm Petri-dish to prevent predation.

All samples were reared in a controlled environmental room (22±1°C, 70-72% RH, and a photoperiod of L12:D12). Image-J software (Abramoff *et al.*, 2004) was used to measure the differences between the total pigmented areas on the pupal dorsal surface for groups O1 and B1. Upon adult emergence, sex was identified according to McCornack *et al.*, (2007).

Furthermore, 100 orange pupae (O2) and 100 dark brown pupae (B2) were collected randomly from the Al-wafra Farm in March 2020. Each pupa was reared in a Petri-dish and kept under field (22.1-23.5±1^{o}C and the relative humidity was 71-73% RH) until the adult stage. The sex of emerged adults was identified following McCornack *et al.*, (2007). For justification, Image-J software was used as above for both groups O2 and B2 for

Data Analysis

All statistical analyses were carried out using the statistical program ‘R’ version 2.8.1 (Ihaka & Gentleman, 1996).

Results and Discussion

The results showed that for laboratory samples, 97orange-colored pupae from group (O1) have developed into females, one pupa gave rise to a male, and two failed to survive (Table 1). On the other hand, 94 males emerged from black pupae and the rest died. Field-reared pupae also displayed a strong color dimorphism in which, 98 black pupae, developed into males, whereas 96 of group (O2) succeeded to grow into females, only one male developed, and two, unfortunately, got dried.

The results of Image-J software are shown in Table (2). For laboratory reared pupae, significant variations were revealed between the mean area of pigment for group (O1) and (B1) (W=0, P<0.0001). For laboratory samples, the mean areas of pigment for group (O1) were (2098.4 ± 64.14μm^2) and it was (86834.87± 26.3 μm^2) for the black pupae (B1). The difference between the two measured means was significant (W=0, P<0.0001).

A significant variation was also obtained between the mean total area of pigment of field groups (O2) and (B2) (W=0, P<0.0001). The mean pigmented area for group (O2) was (18486.58± 54.3 μm^2) and for group (B2) (95725.36± 43.1μm^2). Thus, overall results supported the hypothesis proposed by the current study which is the existence of pupal dimorphism. The quality of environmental cues experienced during immature growth has a great influence on defining the intensity of pigmentation on the external cuticle of pupae (Yamamoto *et al.*, 2011). Hazel and West (2008) reported there is a substantial association between pupal sensitivity and the length of the photoperiod in which short photoperiods produced different coloration irrespective of the back ground or pupation site. Moreover, light stimuli are the most important factor involved in the determination of pupal color (Smith, 1980). For instance, yellow and blue spectral wavelengths are known to determine the formation of green and brown pupae in the Lepidoptera *Pieris rapae, Pieris napi* and *Pieris brassicae,* respectively.Concerning the present study, the results revealed that light was not the fundamental reason for having pupal dimorphism since pupae exhibited two distinctive morphs either the ones that were reared under the laboratory fluorescent light or those that were exposed to sunlight of the field. This outcome made us eliminate the effect of light quality on pigment production.

The influence of substrate features on pupal color has been recognized widely (Huang *et al.*, 2005). Huang *et al.,* (2005) quantified the influence of substrate moisture and grain size on the oviposition of *Anopheles gambiae* (Diptera: Culicidae). They reported that the females of *A. gambiae* often use visual cues to estimate the oviposition qualities with a preference for the dark substrate. The influence texture of the pupation site was reported as well in the butterfly swallowtail, *Papilio machaon* (Hazel, 1996). Nevertheless, the pupal survival rate of desert fleas *Xenopsylla conformis mycerini* and *Xenopsylla ramesis* (Siphonaptera: Pulicidae) was reduced in the sandy substrate as reported by Krasnov *et al.*, (2002). In contrast, the field pupae collected by the present study were found growing on different backgrounds with varied textures and coloration.

Nonetheless, the laboratory samples had been grown in Petri-dish placed on a transparent tray and yet, despite those different backgrounds, pupal dimorphism occurred. Temperature is commonly known as a vital environmental cue playing a substantial role in controlling the development of pupal polymorphism such as in the small copper butterfly, *Lycaena phlaeas daimio* Seitz (Usui *et al.*, 2004). During hot temperatures light-colored pupae are very important in regulating the overall temperature since light colors absorb less heat than dark ones. Xiao *et al.,* (2020) reported that pupal pigmentation can be rapidly induced by temperature, in order to adjust heat absorbance during larval developmental stages and for adapting to the upcoming environmental conditions. In agreement, a study by Knapp and Nedveˇd (2013) reported that there is a linear relationship between the degree of melanization with temperature variations.

Table.1 The outcome adult sex for field and laboratory-reared pupae

Laboratory Reared Pupae	Total Number 200	Color	Survived and The resultant sex		Death
	O1=100	Orange	97	Female	2
			1	Male	
	B1=100	Black	94	Male	6
			0	Female	
Field Reared Pupae	O2=100	Orange	96	Female	3
			1	Male	
	B2= 100	Black	98	Male	2
			0	Female	

Table.2 Mean area of pigment (µm²) on the dorsal surface of laboratory and field-reared pupae measured by Image-J software.

	Color	Mean (µm²)
Laboratory Reared Pupae	Orange (O1)	2098.4±64.14
	Black (B1)	86834.77±26.3
Field Reared Pupae	Orange (O2)	1848.58± 54.3
	Black (B2)	95725.36±34

Fig.1 Pupal dimorphic colours (left is light and right is dark brown)

It was concluded by our study that the degree of melaninization in such cases could be genetically regulated throughout prepupal stages irrespective of the surrounding stimuli. In accordance, Xiao *et al.*, (2020) reported that in *H axyridis*, dopamine which is a melanin precursor is used to regulate the process of melanin production at the early stages of pupation irrespective of environmental factors. Moreover, the current study showed that almost all females tend to have lighter-colored pupae compared with males and the causative factor was explained explicitly by Blanckenhorn *et al.*, (2007). They reported females are characterized by greater growth rates compared with males and the trade-off would be more pronounced particularly in females compared with males due to the highly costly melanin process production. More interpretations regarding the melanin and growth rate trade-offs were declared by

Yasuda & Dixon (2002) and Usui *et al.,* (2004). Studies by Popadić and Tsitlakidou (2021) and Xiao *et al.,* (2020) revealed that melanin production is a common phenomenon in different orders of insects, such as Coleoptera, Lepidoptera, Hemiptera, and Blattodea where it is expressed overthe interplay between regulatory genes rather than external factors. Thus, overall, the evidence regarding the variant pupal morphs appears to support the notion that the fundamental genetic influences were the main determining factor rather than the effect of environmental cues consequences.

In conclusion, our study is the first study that discovered the relationship between pupal pigmentation and the anticipated sex. Thus, we proposed an easy sexing method based on pupal coloration that could help to separate males from females before adult emergence regardless of external influences in *H. axyridis*.

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