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Fluctuating Asymmetry, Morphological Changes and Flight Muscle Ratio in a Vip3A Resistant Sub-population of *Heliothis virescens* (WF06)

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Abstract

Fluctuating asymmetry (FA) of bilateral traits is a measure of developmental instability caused by genetic or environment stressors. The value of FA for a trait depends on its functional importance and it has been argued that the wing characters are more closely associated with the fitness due to their role in locomotion. Flight performance can affect the fitness of individual insects by influencing feeding, predation avoidance, dispersal, inter-male fighting outcomes and mate acquisition. Insect wing lengths are mostly used in FA studies due to ease with which they can be measured accurately. In the present study we have estimated the flight muscle ratio (FMR) and FA in 3 morphological traits of Vip3A resistant and susceptible sub populations of tobacco budworm, *Heliothis virescens* (L.). The Vip3A resistant sub population showed higher FA value for forewing, hindwing and tibia length than the susceptible sub population. The lower FMR observed in the Vip3A-Sel sub-population of *H. virescens* provides indirect evidence that Vip3A resistant insects are poorer fliers as compared with susceptible individuals. The observed results have potential consequences for insecticide-resistance evolution and dispersal.

Keywords: Fluctuating asymmetry; Insecticide resistance; *Heliothis virescens*

Introduction

The measurement of field evolved insecticide resistance can be a useful biomonitoring strategy due to its quick evolutionary response to the insecticide use; the rate of insecticide use being in general directly proportional to the level of insecticide resistance expected (Walker *et al*., 2005). Fluctuating asymmetry (FA) increases when an organism develops under a stressful environment (Hardersen, 2000; Hoffmann *et al*., 2005) and insecticide-resistant insect populations had high levels of FA compared with the susceptible populations (Clarke and Mckenzie, 1987; Clarke *et al*., 2000; Ribeiro *et al*., 2007).

FA is considered to be a measure of developmental instability (Palmer and Strobeck, 1986; McKenzie and Yen, 1995). The assumption behind FA analysis is that the development of both sides of a bilateral symmetrical organism is influenced by identical genes and thus any deviation between the two sides results from developmental perturbation (Clarke, 1998; Jones *et al*., 2005). FA has been shown to increase with genetic and environmental stresses (Vishalakshi and Singh, 2006; 2008) and factors such as increased homozygosity, hybridization, inbreeding, extreme temperatures, chemical exposure and food quality have been linked to FA in morphological traits (Leary and Allendorf, 1989; Mpho *et al*., 2001; Liu *et al*., 2005). The populations that persist under stressful conditions can have mutations in different stress resistance morphological traits and also in the traits genetically correlated with stress resistance (Van Straalen and Timmermans, 2002; Kurbalija *et al*., 2010).

Most insects rely on flight to find food sources, mate or interact socially. When flight performance is compromised these fitness related traits will be impaired (Frazier *et al*., 2008). Flight performance is greatly influenced by body morphology and the ratio of thorax mass to total body mass, the flight muscle ratio (FMR) (Samejima and Tsubaki, 2010). The FMR of insects represents a major allocation of energy and constitutes as much as 55 to 65% of body mass (Marden, 2000; Almbro and Kullberg, 2008).

Heliothis virescens (Fab.) is an important highly polyphagous pest (Neunzig, 1969), which attacks a wide range of food, fiber and oil crops, including cotton in America (Fitt, 1989). This paper examines different morphological characteristics (length of forewing, hindwing and tibia), FA and FMR of Vip3A-Sel and Unsel sub-populations of *H. virescens.*

Materials and Methods

Toxin

Vip3A protoxin was expressed in *Escherichia coli* and purified as described by Yu *et al*. (1997). Lypholized Vip3A

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toxin was obtained from Syngenta (Research Triangle Park, NC) and stored at -80ºC until use.

Insects

A field population of *H. virescens*, (*ca* 300 third to fifth instar larvae) was collected from velvetleaf, *Abutilon theophrasti*, on Wildy Farms, Leachville, MS County, AR, in September 2006, designated WF06 and imported under a Plant Health License (United Kingdom Government, Department for Environment, Food and Rural Affairs) to Imperial College London. In the laboratory the WF06 population was divided in two subpopulations; one subpopulation (Vip-Sel) was selected with Vip3Aa toxin (Syngenta), the other subpopulation was left unselected (Vip-Unsel) (Pickett, 2009; Gulzar *et al*., 2012). The Vip-Sel population was left unselected for 12 generations to avoid maternal effects; at the time of experiment the Vip-Sel resistance ratio was >100.

Measurement of Fluctuating Asymmetry (FA)

One hundred 1st instar larvae of each sub-population were placed on artificial diet. On pupation, pupae were transferred to individual 250 mL plastic cups covered with netting. On emergence, sixty individual adults of each population were randomly selected for the measurement of FA. The insects were frozen at -20ºC and wings and legs detached from the thorax at the point of their attachment and mounted on glass slides. The following traits were measured:

(1). Forewing length of right side (FWLR)
(2). Forewing length of left side (FWLL)
(3). Hindwing length of right side (HWLR)
(4). Hindwing length of left side (HWLL)
(5). Tibia length of meta leg of right side (TBLR)
(6). Tibia length of meta leg of left side (TBLL).

A dissecting stereo microscope with eyepiece graticule (units 1-100 µm) was used; measurements were taken through the right eyepiece only to avoid distortion from change of angle (Peck, 2009).

Flight Muscle Ratio (FMR)

The head, thorax and abdomen from 60 adults (30 male and 30 female) selected from each sub-population were separated and dried for 24 h at 55ºC, then weighed using a microbalance. The FMR was calculated as follows:

FMR = Dry thorax mass/Dry body mass

Analysis of Fluctuating Asymmetry (FA)

Measurement error (ME) can account for variation in asymmetry, and repeat measurements are required to ensure that FA is detectable and that there is no effect associated with the directional asymmetry (Vishalakshi and Singh, 2008). In order to estimate the ME, 20 adult insects were randomly taken from the culture and each insect trait was measured twice. The significance of ME was assessed using a two-way mixed model ANOVA, where sides were entered as fixed factors and individuals as random factors (Vishalakshi and Singh, 2008). FA was calculated for each trait of every individual using the indexes:

FA = [R-L]

Where, R is the value of trait on right side and L is the value of trait on the left side.

The resulting measurement was added to the data set.

Statistical Analysis

Data were analyzed in R version 2.9.0 (R Development Core Team, 2009). For FA, a two-way ANOVA test of side (fixed) × individuals (random) with repeated measurements of each side was used to assess the magnitude of measurement error. The Shapiro-Wilk test was applied to check the normality of the trait where the error mean square is larger than side x individual mean square (Peck, 2009). FA values, FMR and length of morphological traits were analysed by ANOVA.

Results

FA Data Validation

A two-way ANOVA with replicated measurements of each side, indicated that the mean square for error was not significantly smaller than side × individual (non-directional asymmetry), with two out of the six traits having larger errors than side × individual (Table 1). This meant the ME for these three traits was of concern for the analyses of FA in the full data set as it appeared ME (measurement error) could be larger than the calculated FA. However, despite the implications of high ME in these traits, directional asymmetry could be ruled out as there was no significant difference between right and left measurement 'sides' for any of the traits (ANOVA p>0.05, df = 1, n = 20) and anti-symmetry could be ruled out as the data did not depart from normality (Shapiro-Wilk test, p>0.05). It can therefore be assumed that the asymmetry occurring is due to FA.

Mean Values of Different Morphological Traits

The mean ± SE of different morphological traits of Vip3A-Sel and Unsel adults are shown in Table 2. Mean FWLR (F= 11.51, df 1,57, p<0.01) and FWLL (F = 19.57, df 1,57, p<0.001) were significantly smaller in the Vip3A-Sel compared with the Unsel sub-population. The mean HWLR (F = 10.78, df 1,74, p<0.01) and HWLL (F = 20.93, df 1,74, p<0.001) were significantly smaller in Vip3A-Sel compared with Unsel. The mean tibia length of right side (F= 7.82, df 1,67, p<0.01) and left side (F = 14.50, df 1,74, p<0.001)

Table 1: Two-way ANOVA (side x individual) performed for each trait to assess measurement error (ME) for a sub-sample of 20 specimens from Vip3A-Sel and Unsel sub-populations of *Heliothis virescens* measured twice

Trait		Mean Square (ANOVA)			
		Sides	Individual	Side x individual	ME
Forewing	Unsel	0.006	4.93	0.01	0.0 1
Forewing	Vip3A-Sel	0.124	5.298	0.004	1.20
Hindwing	Unsel	0.180	2.25	0.15	0.11
Hindwing	Vip3A-Sel	0.080	7.100	0.20	0.10
Tibia	Unsel	0.122	.508	0.01	0.01
Tibia	Vip3A-Sel	0.028	0.321	0.001	0.04

Table 2: Mean (mm ± SE) morphological traits of Vip3A-Sel and Unsel sub-populations of *Heliothis virescens.* FWLR = forewing length of right side; FWLL = forewing length of left side; HWLR= hindwing length of right side; HWLL= hindwing length of left side, TBLR= tibia length of right side; TBLL= tibia length of left side

	FWLR	FWLL	HWLR	HWLL	TBLR	TBLL
Unsel	14.61 ± 0.09^a	14.41 ± 0.10^a	10.67 ± 0.07^a	10.50 ± 0.08^a	3.43 ± 0.03^a	3.36 ± 0.03^a
Vip3A-Sel	14.28 ± 0.06^b	13.91 ± 0.08^b	10.34 ± 0.06^b	10.02 ± 0.06^b	3.32 ± 0.02^b	3.21 ± 0.02^b

Mean values (±) followed by a different letter are significantly different (P< 0.01) between the two sub-populations

were significantly smaller in Vip3A-Sel compared with Unsel.

Overall Trend for Fluctuating Asymmetry (FA)

The ANOVA for FA of *H. virescens* indicated significance differences between the two sub-populations. The mean FA value of forewing in Vip3A-Sel was significantly greater compared with Unsel (Fig. 1a; F= 7.17, df 1, 59, p<0.001). The mean FA value of hindwing in Vip-Sel was significantly greater compared with Unsel (Fig. 1b; F=32.411, df 1, 74, p<0.001). The mean FA value for tibia in Vip3A-Sel was significantly greater compared with Unsel (Fig. 1c; F = 16.42, df 1, 67, p<0.001).

Flight Muscle Ratio (FMR)

The FMR of the Vip3A-Sel sub-population was significantly less compared with Unsel (Fig. 1d; F = 17.78, df 1,198, P < 0.001).

Discussion

The value of FA for a trait depends on its functional importance (Vishalakshi and Singh, 2009) and it has been argued that the wing characters are more closely associated with the fitness due to their role in locomotion (Clarke *et al*., 2000). Insect wing lengths are mostly used in FA studies due to ease with which they can be measured accurately (Mpho *et al*., 2001). Stress can disrupt the developmental stability of a trait and increase the FA level but traits may vary in their sensitivity to specific stress factors (Polak *et al*., 2002). It is also reported that the magnitude of the stress over the time may have different effect on the FA value as well as on the fitness (Van Dongen, 2006).

The present study suggests that FA can be linked with resistance to Vip3A in *H. virescens*, with FA values for forewing, hindwing and tibia length being greater in the Vip3A-Sel sub-population than the Unsel sub-population. Despite the potential of FA as a method of studying fitness effects; evidence linking FA with insecticide resistance has been limited to two species, the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (Clarke and McKenzie, 1987; Clark *et al*., 2000) and the maize weevil, *Sitophilus zeamais* (Motsch) (Coleoptera: Curculionidae) (Ribeiro *et al*., 2007). The present results are similar to studies with *L. cuprina*, where insects resistant to organophosphates, had higher FA values and reduced fitness compared with susceptible insects (Clarke *et al*., 2000). Studies have also shown higher FA in the mosquitoes treated with the organophosphate insecticide temephos compared with control insects (Mpho *et al*., 2001). While studies showed that laboratory and field populations of *H. armigera* reared on Bt cotton displayed increased FA levels compared with populations reared on non-Bt crops (Li *et al*., 2004) and Liu *et al.* (2005) reported similar findings for the aphid *Aphis gossypii* (Glover) feeding on Bt compared with non-Bt cotton.

FA can be affected by continuous selection pressure. An initial selection with organophosphates led to higher levels of FA compared with unselected populations in *L. cuprina* (McKenzie and Clarke, 1987) but continued selection led to a return to the FA levels found in the susceptible populations (McKenzie and O'Farrell, 1994). Ribeiro *et al.* (2007) have reported that in the maize weevil, *Sitophilus zeamais* (Motsch) (Coleoptera: Curculionidae) resistant to deltamethrin had a lower FA value compared with susceptible insects. This may have been due to the evolution of a modifier allele that enhanced the fitness of resistant insects (Ribeiro *et al*., 2007). Investigations of FA in *L. cuprina* have shown a series of distinctive qualitative and quantitative shifts in FA between resistant and susceptible strains. McKenzie and Clarke (1987) found that wild resistant populations that had been exposed to the organophosphate insecticide diazinon for many generations had no significant difference in FA compared with

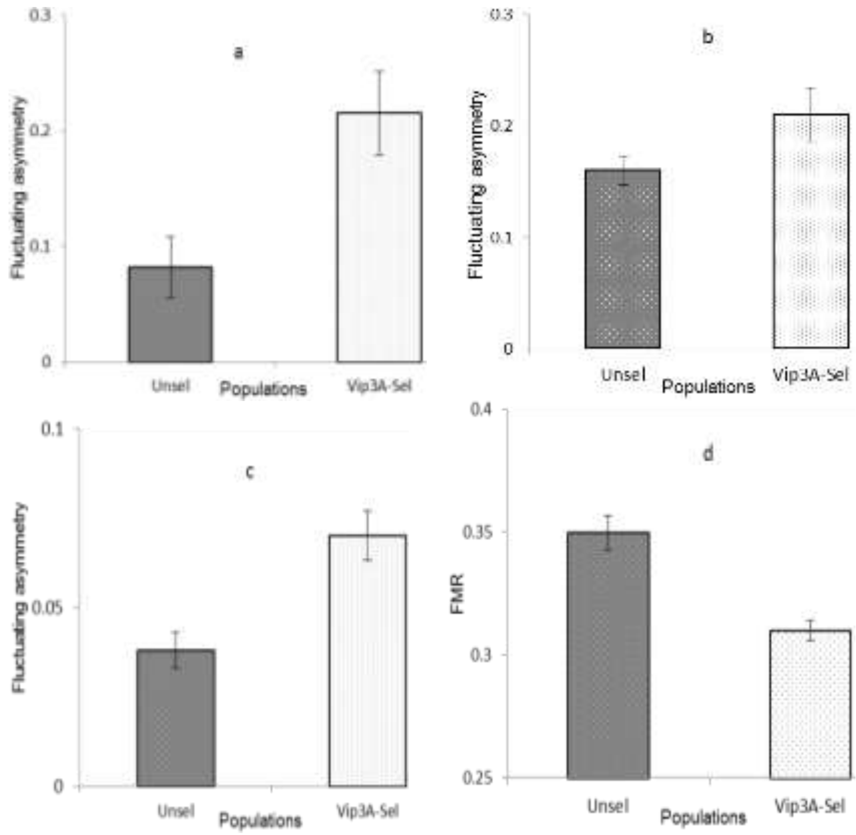

Fig. 1: Fluctuating asymmetry for (a) forewing length (b) hindwing length (c) tibia length and (d) Flight muscle ratio (FMR) in Unsel and Vip3A-Sel sub-populations of *H. virescens*

susceptible populations. Backcrossing indicated the presence of a modifier allele that both ameliorated the fitness costs of resistance and returned FA levels to those comparable with susceptible populations (McKenzie and O'Farrell 1994). These experiments revealed that the same modifier allele was responsible for the amelioration of fitness costs and FA in *L. cuprina* independently selected for malathion resistance (McKenzie and O'Farrell, 1994). In addition, the modifier allele was specific to modifying the asymmetry repercussions from insecticide exposure and analysis showed the allele to be dominant and independent acting against other stressors such as temperature and population densities (Freebairn *et al*., 1996).

Flight performance can affect the fitness of individual insects by influencing feeding, predation avoidance, dispersal, inter-male fighting outcomes and mate acquisition (Marden, 1989; Chai and Srygley, 1990; Coelho and Holliday, 2001; Marden and Cobb, 2004; Almbro and Kullberg, 2008; Samejima and Tsubaki, 2010). A decrease in the flight activity in butterflies (*Colias* spp. and *Pontia* spp.) was reported by Kingsolver and Srygley (2000) after an experimental reduction of FMR by 10-17%. Interspecific studies of tropical butterflies revealed that species with higher FMR showed faster flight and better manoeuvrability than the species with lower FMR (Pinheiro, 1996; Kingsolver and Srygley, 2000; Almbro and Kullberg, 2008). The lower FMR observed in the Vip3A-Sel sub-population of *H. virescens* provides indirect evidence that Vip3A resistant insects are poorer fliers compared with susceptible individual.

In the present study, the length of right and left forewing, right and left hindwing, and right and left tibia was smaller in each case for the Vip3A-Sel sub-population compared with the Unsel sub-population. These results were similar with earlier in which the length of first, second and third instar was significantly smaller in a population of the whitefly *Bemisia tabaci* (Genn.) (Hemiptera: Aleyrodidae) selected with thiamethoxam compared with an unselected population (Feng *et al*., 2009).

Further studies are required to investigate the molecular genetic changes responsible for the observed variations in morphological traits, FA value and FMR in Vip3A-Sel *H. virescens*, and their stability and significance in relation to the performance (fitness) of resistant compared with susceptible insects.

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