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Thermotolerance of *Drosophila* hybrids: a new mode of adaptation in extreme climatic conditions.

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Abstract

Hybridization is not common in natural *Drosophila* populations. Trait expression and ecological performance of hybrids determine the consequences of hybridization such as degree and direction of gene flow or generation of phenotypic novelty. We investigated plastic effects on thermoresistance traits by growing parental species namely, *Drosophila jambulina* and *Drosophila punjabeensis* and their hybrids, at four growth temperatures. Hybrids show increased variation in thermotolerance traits than their parental species. Hybrids show higher plasticity as well as cumulative fitness under variable environmental conditions. Acclimation effects were also significant in hybrids concluding adaptive potential. Results suggest that hybridization increases genetic variation that produces adaptation to new environments. We conclude that plasticity studies on hybrids and their progenitors are useful for testing basic predictions about evolution, as well as for understanding the evolutionary significance of hybrids. Key words: sibling species; *Drosophila jambulina*; *Drosophila punjabeensis*; hybrid zone; genetic variation; fitness; acclimation

Introduction

Evolutionary significance of natural interspecific hybridization has gained much attention in recent years. In hybrids, trait expression and their ecological performance depends on the genetic control of the traits and the nature of genetic differences in their parental species. The outcome of natural hybridization varies in different taxonomic groups, but it has played a part in the evolution of large number of contemporary and

extinct organisms (Arnold, 1997). There are studies that have argued for an important role for hybridization in the evolution of species (*e.g.*, Anderson and Stebbins 1954). Many attempts have been made to hybridize different species of *Drosophila*, but most of them were unsuccessful (Sturtevant, 1920), except in a few cases, *e.g.*, *D. serrata* and *D. birchii* (Ayala, 1965). However, the majority of species do not hybridize in nature, but in laboratory they do so (Bock, 1984). Interspecific hybridization often leads to embryonic or adult lethality and reduced viability or fertility of hybrids (Sperlich, 1962; Llopert *et al.*, 2005) due to accumulation of genes that cause recessive incompatible epistatic interactions between the parental species (Dobzhansky- Muller model, Presgraves, 2003). Hybridization can also provide insights into plasticity as a mechanism for evolutionary change.

For ectothermic organisms, differences in thermal resistance match geographical distribution patterns of species, *i.e.*, a high level of cold resistance is linked with higher latitudes while a reverse trend occurs for heat resistance (Hoffmann, Sorensen, and Loeschke, 2003; Chown and Nicolson 2004). There are studies that abound on consequences of hybridization on speciation, formation of novel populations, fitness of hybrids and parents, genetic variation (Hatfield and Schlüter, 1999; Schartl *et al.*, 1995; Norris *et al.*, 1999). Comparison of plasticity of hybrids and their parents will provide insights about persistence and adaptability of hybrids. Genetic variations for heat and cold resistance have been examined by comparing species and populations as well as on the basis of laboratory selection experiments (Gilchrist and Huey, 1999; Hoffmann, Sorensen, and Loeschke, 2003). Thus, there are heritable genetic variations in thermoresistance traits, which confer adaptations to climatic conditions.

Consideration of closely related species and their hybrids is a way of examining genetic divergence on a longer time scale (Hercus and Hoffmann, 1999). Hybridization studies would be instrumental in describing how a species increases its ecological tolerance and expansion of geographical range. Analysis of variance in hybridizing species will provide necessary evidence for natural selection that helps a species in adapting to new environments. Further, plastic studies are essential in hybrids as their fitness relative to the parents may vary depending on the environment (Mercer *et al.*, 2006; Darwish and Hutchings, 2009). Analysis of reaction norms is important for across- environment studies (Morris *et al.*, 2011).

D. jambulina and *D. punjabeensis* (Prashad and Paika, 1964) are Asian endemic *Drosophila* species. These two sibling species present an ideal situation for looking at the effect of plasticity in hybrids. In both species light allele is dominant over dark allele, but only the later one shows plasticity to some extent. In spite of huge work on hybridization and phenotypic plasticity, there are very few studies regarding phenotypic plasticity of hybrids in extreme conditions. This study addresses the influence of plasticity on hybrid fitness and adaptability. To study the potential effect of natural hybridization and phenotypic plasticity in the genus *Drosophila*, this study focuses on the conditions that involve natural hybridization between these two sibling species. We used morphometric and physiological approaches to quantify effects of hybrid plasticity in drosophilids from different sites. Comparative analysis was used to study what role hybrid phenotypic plasticity plays in adaptation to extreme conditions. Fitness analysis related to thermotolerance assays was performed to examine significance of hybridization and capability of evolutionary adaptation. We tried to score out whether plasticity for heat and cold traits differ between the two parental species and their hybrids. Specifically, we ask how plasticity and acclimation capacity generated due to effect of hybridization help in adaptation at a broader thermal range. Both the parent species have different thermal limits and occur in different biogeographical regions. Plasticity and acclimation capacity generated due to effect of hybridization help in adaptation in a broader thermal range.

Material and Methods

Collection and cultures

D. jambulina and *D. punjabeensis* are sibling species inhabiting different biogeographical regions. The former one is a tropical species and the latter inhabits subtropical localities. *D. punjabeensis* exhibits considerable variation in abdominal variation. Both these species are very similar and were confused with each other. Also, their hybrids were often misleading one who separates the mixed wild stocks. Both exhibit color dimorphism, having light allele dominant over darker one. The collections of sympatric populations of *D. jambulina* and *D. punjabeensis* were made in a single trip during pre-winter months from six altitudinal

sites. From each site, about 200-300 flies were collected using net sweeping and bait trap methods. Isofemale lines from wild caught individuals were maintained at 25°C on standard cornmeal yeast agar medium.

Isofemale lines were distinguished among *D. jambulina*, *D. punjabeensis*, and hybrids. To generate hybrid populations in the laboratory, crosses were undertaken by single pair mating using true breeding isofemale lines from both species in both directions. Morphological characters were used for confirmation of hybrid status. An equivalent number of parental lines of the two species were also set up to serve as controls. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in).

Trait measurement

Thermotolerance of hybrids and their parents were measured in both control as well as acclimated groups. For thermotolerance traits and acclimation assessment, heat knockdown and chill coma tolerance were preferred, because these are ecologically relevant and confer repeatability. Effects due to age, sex, anesthesia, ambient room temperature, and thermal conditions of assay vials were controlled. Seven day old flies were aspirated and introduced to assay vials, which were pretreated at experimental temperatures for 6 hours so as to minimize effects due to thermal variations. For all traits, measurements were made in a thermocontrolled room at 21°C.

We analyzed plastic effects on thermoresistance traits by growing parental species and their hybrids at four growth temperatures (17 to 28°C). All experiments were performed on adult flies selected randomly from each iso-female line. For examining growth range (lower and upper developmental temperature), eggs of *D. punjabeensis*, *D. jambulina*, and their F1 hybrids were transferred to 14, 15, and 16°C for lower and 28, 29, 30, and 31°C for the upper limits. Temperatures at which egg to adult development occurs at the both ends were considered as lower and upper developmental temperatures.

For acclimation effects, adult acclimation treatment by exposing adults at high and low temperatures for varying durations of time was undertaken. Experiments were carried out with females from isofemale lines of both the parental species and their hybrids. Acclimation temperatures were similar to those that the parental forms encounter in the field. Adults were exposed to high and low temperatures for 2, 4, 6, 8, and 10 days and were transferred to growth temperature for 24 hours before experimental analysis.

Fitness analysis was performed by mimicking natural habitat conditions of the two parental species. For estimating fitness, survival rate (w) was calculated as the proportion of group (*D. punjabeensis*, *D. jambulina*, or hybrid) that survive after selection in two habitat conditions by dividing the number of surviving individuals by total number of individuals of that group. Secondly, fitness was computed by dividing each genotype survival rate by maximal survival rate. Finally, cumulative fitness was calculated.

Statistical analysis

Since the thermoresistance traits showed high repeatability across G1 and G2 generations, such data were pooled. For all the traits, isofemale line means ($n = 20$) along with s.e. or s.d. were used for illustrations and tabular data. For trait variability analysis, ANOVA helped in comparing F values and their percent variation contribution. Acclimation effects were compared through t-test (Zar, 1996). For estimating hybrid superiority, fitness as well as cumulative fitness was calculated. Statistical calculations and illustrations were made with the help of Statistica™ 5.0.

Results

D. punjabeensis and *D. jambulina* are sibling species that are adapted to different biogeographical regions and are thermal specialists. Adults show only slight morphological differences and are hard to distinguish. For six localities, flies were identified as hybrids and pure species on the basis of morphological differences (Table 1). t-test values indicate that there were significant morphometrical differences between the two parent species. Trait values in hybrid individuals were intermediate of their parents. Table 2 shows average hybrid index and percentage of pure species from the six altitudinal collection sites. Hybrids were found in climatic zones where temperature fluctuations are high. Analysis of thermal range clarifies that

hybridization increases growth range of hybrid individuals (Figure 1). Increase in thermal range of hybrid will help in adaptation to wider range of growth temperatures than their parental species.

Table 1. Mean (\pm S.D.) values for various morphometrical traits and thermal range in *D. punjabeensis*, *D. jambulina* and their hybrids. For significance level between hybridizing parent species, student's t- test values were used.

Trait	Parental species			t-test	Hybrid
	<i>D. punjabeensis</i>	<i>D. jambulina</i>			
Wing length (mm)	2.38	2.05		**	2.20
Thorax length (mm)	1.05	1.00		*	1.01
Wing width (mm)	0.98	0.77		**	0.85
Body weight (mg/fly)	0.000815	0.001053		***	0.000872
Eye spot	0	3		***	1
Sex comb teeth (no.)	24,19	24,15		ns*	24,17
Thermal range (°C)	15-28	17-31		**	15-29

Table 2. A comparison of phenotypic plasticity of thermotolerance traits in two *Drosophila* species and their hybrids. Plasticity was quantified as coefficient of variation (CV) across complete thermal range; and on the basis of t- test acclimation capacity was compared.

** = $p < 0.01$, *** = $p < 0.001$; ns = nonsignificant.

Species	Heat Resistance		Cold Resistance	
	CV	t-test	CV	t-test
<i>D. punjabeensis</i>	23.51	9.18 **	20.32	11.15 **
Hybrids	39.14	23.08 ***	43.57	46.15 ***
<i>D. jambulina</i>	1.80	0.71 ns	4.21	1.13 ns

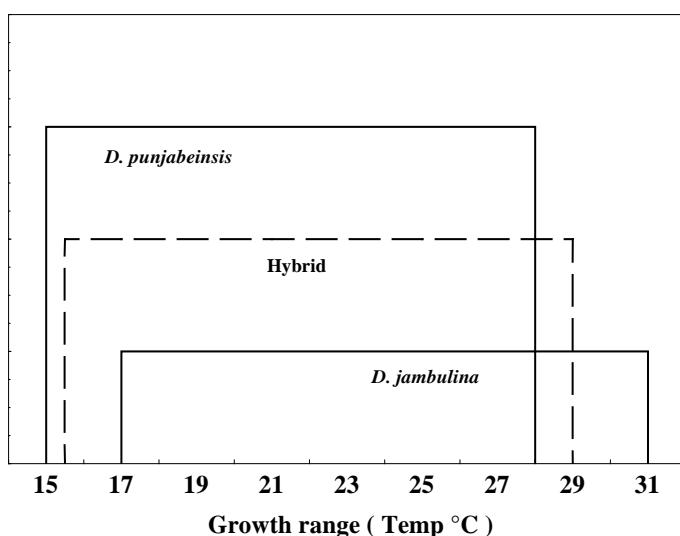


Figure 1. Developmental growth range of *D. punjabeensis*, *D. jambulina*, and their hybrids.

Thermotolerance in hybrids and their parents

We assessed heat and cold tolerance in *D. punjabeensis*, *D. jambulina* and their hybrids. There are significant differences between the two parental species based on t-test ($p = 0.001$), whereas the hybrids have intermediate values. Also, *D. punjabeensis* shows significant geographical differences in heat and cold tolerance. We assessed plastic effects due to developmental temperatures (17 to 28°C) on thermotolerance traits. Hybrids show increased variation in thermotolerance trait than their parental species as indicated by the results of ANOVA (Table 3). Figure 2 illustrate contrasting differences in the coefficient of variation (CV) as a measure of plasticity of thermoresistance traits of *Drosophila* species and their hybrids. Plasticity is moderate in *D. punjabeensis*, but *D. jambulina* lacks plasticity for thermal variables while hybrids have shown a high level of plasticity. These varying plasticity levels correspond with species survival in different climatic (temperate vs. tropical) zones.

Table 3. Effect of thermal acclimation on heat and cold resistance in *D. punjabeensis*, *D. jambulina* and their hybrids. Significance level between control and acclimated groups are tested using student's t-test. F- statistics values are given from analysis of variance results

Species	Heat resistance			Chill coma recovery		
	Non-acclimated	Acclimated	F- value	Non-acclimated	Acclimated	F- value
<i>D. punjabeensis</i>	8.21 ± 1.02	10.23 ± 0.87	4.63*	15.01 ± 2.00	12.68 ± 1.02	5.18*
<i>D. jambulina</i>	19.25 ± 0.98	20.32 ± 0.58	1.28 ns	27.02 ± 1.85	26.59 ± 0.98	2.14 ns
Hybrids	15.21 ± 2.12	21.02 ± 3.56	47.89***	20.25 ± 3.25	12.43 ± 4.11	56.87***

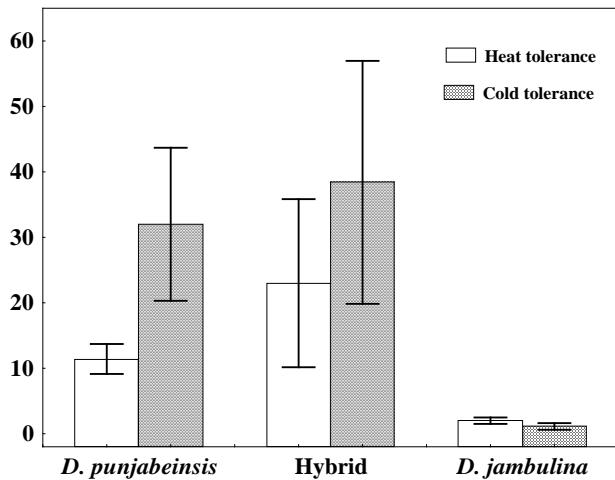


Figure 2. Bars showing coefficient of variation (CV) for thermoresistance traits due to changes in growth temperatures. SD lines demonstrate acclimatory capability of parents and their hybrids grown at 21°C and acclimated to upper and lower thermal environments.

Acclimation to thermal environments

Heat and cold tolerance were significantly affected by acclimation in hybrid individuals followed by *D. punjabeensis*, whereas *D. jambulina* lacks acclimation effects (Figure 3). Acclimation to lower temperature significantly increases cold tolerance and decreases heat resistance in hybrids. Comparison of t-test values for control (non- acclimated) and acclimated flies shows that hybrids have high potential to acclimate or adapt to new thermal environment. *D. jambulina* lacks acclimatory capacity (t-test value = 0.71). Analysis of variance (ANOVA) indicates significant F- statistics differences on thermoresistance due to acclimation in hybrids (Table 4). Acclimation, therefore, increases resistance towards thermal stresses.

Parents vs. hybrids fitness

Hybrids are generally considered as less fit as compared to their parents. Study of survivorship after exposure to heat and cold was conducted for analyzing fitness. Hybrids show significantly higher overall fitness than their parental species in variable environments. Table 4 shows highest cumulative fitness for

hybrid individuals under a series of selective events. These results provide evidence for increased hybrid fitness.

Table 4. Hybrid advantage calculated as survival under different conditions during two selective environments.

Species	W_1 (Cold)	W_2 (Hot)	$W_{\text{cumulative}}$	W_{relative}
<i>D. punjabeensis</i>	1	0.23	0.23	0.51
Hybrids	0.63	0.71	0.45	1.0
<i>D. jambulina</i>	0.29	1	0.29	0.64

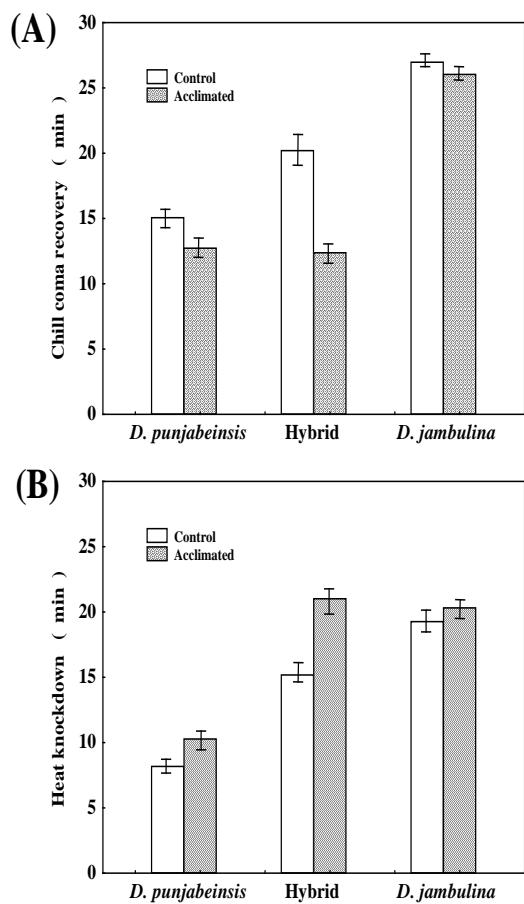


Figure 3. Relative changes in chill coma recovery (A) and heat knockdown (B) as a result of acclimation to different thermal environments in *D. punjabeensis*, *D. jambulina*, and their hybrids.

Discussion

D. punjabeensis and *D. jambulina* have different ecological requirements and hence are adapted to different habitats. Temperature is the main limiting factor; the former can cope with lower temperature and thus dominates in montane localities. In habitats with intermediate temperatures, both of these coexist as sympatric populations. Habitats with fluctuating temperatures are the natural laboratories for hybridization. Due to high morphological similarities between these two species, separations between parental species and their hybrids were based on differentiating characteristics based on results of crosses from laboratory hybrids. Hybridization between sibling species has been recognized to increase genetic diversity, often resulting in trait values of hybrid individuals exceeding their parents (Anderson, 1949; Ellstrand and Schierenbeck 2000; Bruce and Arnold 2001). For species having low genetic variation, hybridization can provide sufficient variation for adaptive evolution (Ellstrand and Schierenbeck 2000;

O'Hanlon *et al.*, 1999). Consistent with previous studies, we found an increase in thermal tolerance variability in hybrids of *D. punjabeensis* and *D. jambulina*.

Insects can cope with seasonal environments through phenotypic plasticity, which allows a single genotype to produce different phenotypes (Holloway *et al.*, 1997; DeWitt and Scheiner, 2004). Adaptive plastic responses for thermotolerance traits have been analyzed on the basis of short term exposures to sub-lethal conditions (hardening) and long-term exposure to conditions in the viable thermal range (acclimation) in different *Drosophila* species (Hoffmann, Sørensen and Loeschke, 2003; Chown *et al.*, 2009). Plasticity can facilitate tolerance to a broad range of ecological conditions and spread of species to new environments. Hybrids of *D. jambulina* have high levels of thermal plasticity (measured as CV) and performed consistently better than their parents.

The degree to which an organism can acclimate is dictated by their phenotypic plasticity or the ability of an organism to change or adapt to a certain environment. Out of the two studied *Drosophila* species, *D.*

jambulina lacks acclimation capacity and thermal plasticity. Acclimation capacity of *D. punjabeensis* represents an adaptation to a wide range of ecological conditions. Acclimation capacity of an individual might provide an understanding of how an organism can respond to an increase or decrease of habitat temperature. Studies related to hybridization effect on acclimation capacity and its association with habitat expansion have not been considered yet. Physiologists have assumed acclimation as a form of phenotypic plasticity that enhances performance of a species or individual in a particular environment (Hochachka and Somero, 2002; Prosser, 1986). Studies on hybrids of *D. serrata* and *D. birchii* provides no evidence that hybridization increases evolutionary rates and adaptation (Hercus and Hoffmann, 1999). In contrast, the results of the present study indicate that hybrids have increased variation and acclamatory capacity that leads to an increase in adaptability of a species to new ecological conditions. Acclimation is a source of phenotypic adaptation resulting in widening of regulatory range of a species (Horowitz, 2001). In the present study, results of acclimation experiments were in favor of previous reports and indicate adaptive significance of hybridization.

Significant differences were obtained in fitness of hybrid and parental species. Thermal conditions change over geographical regions. Fitness of a species is influenced by both genetic and environmental conditions; hence, fluctuation in environmental conditions can act as selective regimes, which can produce cumulative hybrid advantage. Under one or the other single selective events, parental species are more successful as they are genetically better adapted to that environment. Fitness estimation of few hybrid genotypes is equal to or greater than those of their parents suggesting a significant role of natural hybridization in adaptive evolution (Arnold and Hodges, 1995). The present study favors that hybridization cause an addition to genetic variability that can facilitate range and/or habitat expansion of sibling species.

Hybridization in these two *Drosophila* species is, therefore, helpful for *D. jambulina* (a warm specialist species) to extend its adaptive potential, acclimation capacity, and plasticity. This expansion involved physiological adaptation and tolerance to extreme thermal conditions. The genetic variation that is necessary to increase ecological tolerance of *D. jambulina* has come from *D. punjabeensis*. We predict that species with low potential towards adaptation to fluctuating environments can enhance their tolerance and adaptive capability via hybridization. Environmental conditions fluctuate in time and change over geographical regions. Both the genetic and environmental factors influence the fitness and adaptive potential of an organism. Thermal tolerance traits have been demonstrated as reliable tools for demonstrating climatic adaptations in populations and species of *Drosophila* (David *et al.*, 2003; Gibert *et al.*, 2001). In the present study, we used plastic responses, acclimation capacity and fitness under variable environments to assess the effect of hybridization in adaptation and improvement of survival as a measure of sibling rescue under drastic environmental conditions. Hybrid individuals are more plastic than their parental species and have increased developmental range at both extremes. Analyses of acclimation responses to temperature are more and occur quickly in hybrids. Results showed that under fluctuating environmental conditions, hybrids are more successful than their parental species as the cumulative fitness is higher for hybrids. Our results lead to conclude that hybridization in sibling species is adaptive and can lead to evolution. Therefore, hybridization between two species can be a potential stimulus for adaptation and rapidly create evolutionary novelties.

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Polyphenols as treatment at the intersection of environmental and genetic causes of Parkinson's disease in a *LRRK2* model.

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Abstract

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects between 4 and 5 million people worldwide. The etiology of PD is both environmental and genetic. Thus far, research has shown that the reduction of environmentally triggered reactive oxygen species (ROS) levels can reduce parkinsonian symptoms in several animal models (Bonilla-Ramirez *et al.*, 2011). Furthermore, several genetic mutations including *LRRK2* (Leucine-rich repeat kinase 2) that lead to impaired mitochondrial function have been shown to impact Parkinson disease onset (Guo, 2012). We have used a fly *LRRK2* knockout model to study the role of environmental factors in PD development. *LRRK2* knockout flies were fed an antioxidant solution of polyphenols (propyl gallate, epicatechin, gallic acid, and epigallocatechin gallate) every 5 days. Polyphenol-fed flies and controls were examined using several measures of parkinsonian symptoms. Survival numbers, climbing ability, and dopamine immunohistochemistry were performed on flies at several times post-eclosion. *LRRK2* knockout flies fed with polyphenols did not show an altered lifespan, but showed a decrease in motor impairments. Treatment with polyphenols also decreased dopaminergic neuron degeneration as compared to control. It may be that polyphenols can effectively combat increased ROS due to impaired mitochondrial function in *LRRK2* mutants.

Introduction

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects motor movement due to death of dopaminergic neurons in the substantia nigra pars compacta. Approximately 50,000 people in the U.S. are diagnosed with PD annually, and between 4 and 5 million people suffer from Parkinson's disease worldwide (Michael J. Fox Foundation, 2012). The major symptoms of PD include tremors, trembling, rigidity of limbs, slowed movement, poor posture, and issues with balance. There is no known diagnostic test or a cure for PD.

PD has both environmental and genetic origins and has been associated with defects in mitochondrial function. Defective mitochondria produce more reactive oxygen species (ROS), which are associated with aging, age-related disease, and neurodegeneration. Certain industrial environmental agents and agricultural chemicals, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or rotenone, produce PD-like symptoms by damaging mitochondria, which then produce increased levels of ROS (Jimenez-Del-Rio *et al.*, 2010). Heavy metals and paraquat (PQ) generate ROS and, in turn, parkinsonian symptoms (Jimenez-Del-Rio