EFFECTS OF TOXICANTS, TEMPERATURE, AND RESISTANCE ON METABOLISM AND GAS EXCHANGE PATTERNS OF THE BEET ARMYWORM, *SPODOPTERA EXIGUA* (HÜBNER), AND THE GERMAN COCKROACH, *BLATTELLA GERMANICA* (LINNAEUS)

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Beatrice Nuck Dingha

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DISSERTATION ABSTRACT

EFFECTS OF TOXICANTS, TEMPERATURE, AND RESISTANCE ON METABOLISM AND GAS EXCHANGE PATTERNS OF THE BEET ARMYWORM, *SPODOPTERA EXIGUA* (HÜBNER), AND THE GERMAN COCKROACH, *BLATTELLA GERMANICA* (LINNAEUS)

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Components of the respiratory physiology and water relations of *Bacillus thuringiensis* Cry1C resistant and susceptible beet armyworms, *Spodoptera exigua,* and pyrethroid resistant and susceptible German cockroaches, *Blattella germanica*, were investigated. Standard metabolic rates of third and fifth instar larvae and 1 to 7 day old pupae of *S. exigua* and adult *B. germanica* were determined using closed-system respirometry. $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$) of third instar resistant larvae reared continuously on toxin (CryonT) were significantly greater than resistant third instar larvae reared on toxin for 5 days and reared thereafter on untreated diet (Cry5dT), resistant larvae reared

on untreated diet (CryReg), and the susceptible parental strain (SeA) reared on untreated diet. No difference in metabolic rates among treatment groups was detected for fifth instar larvae. CryonT larvae and pupae weighed significantly less than those receiving other treatments. One day-old pupae of all treatment groups exhibited a high $\dot{V}_{O_2}$. Metabolic rates of all treatment groups declined between days 2 and 4 then increased between days 4 and 7. Overall $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}) was not significantly different between strains of *B. germanica* but different strains reacted differently to increasing temperature. Mean cockroach body mass differed significantly between strains and $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}) scaled with temperature and mass in all strains.

Resistant and susceptible *S. exigua* pupae and adult *B. germanica* exhibited a discontinuous gas exchange cycle (DGC), which was characterized using flow-through respirometry at 10-35^{o}C. DGC was exhibited mostly at 10^{o}C; frequency of cyclic CO_2 release increased with increasing temperatures. There was no clear distinction between the closed and flutter phases in most DGCs, from *B. germanica*. The three phases were distinct in *S. exigua*. Respiratory water loss was 4.5$\pm$1.3%, 2.1$\pm$2.4%, 3.4 $\pm$ 1.9% and, 4.4 $\pm$2.2% for toxin exposed *S. exigua*, unexposed *S. exigua*, insecticide exposed *B. germanica* and unexposed *B. germanica*, respectively. Toxin exposed *S. exigua* pupae had a significantly greater cuticular permeability (26.01$\pm$1.9 μg cm^{-2} h^{-1} $mmHg^{-1}$) than unexposed pupae (9.64$\pm$0.9 μg cm^{-2} h^{-1} $mmHg^{-1}$). The cuticular permeability of *B. germanica* was 2.26 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the resistant strain and 3.42 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the susceptible strain. In both strains of *S. exigua* and *B. germanica* cuticular transpiration (>93%) far exceeds respiratory transpiration.

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CHAPTER 1: LITERATURE REVIEW

Insecticide resistance

One of the major problems with insect control is the evolution of resistance to insecticides. Insecticide resistance is the inherited ability of an individual insect to survive exposure to a concentration of insecticide that is generally lethal to susceptible insects. The first widely recognized case of pesticide resistance among agricultural arthropod pests was reported in the early 1900s, when lime sulfur failed to control the San Jose scale, Quadraspidiotus perniciosus (Comstock) (Homoptera: Diaspididae), in Washington State. Later in 1946, houseflies, Musca domestica (L.) (Diptera: Muscidae), were discovered to be resistant to DDT in Sweden. Since then, more than 500 species of arthropod pests have evolved resistance to one or more major groups of insecticides, including organochlorines, organophosphates, carbamates, and synthetic pyrethroids (Georghiou, 1986). In 1979, the United Nations Environmental Program declared pesticide resistance one of the world's most serious environmental problems. Its seriousness to the environment stems from the fact that after resistance to an insecticide develops there is application to the environment of new and potentially dangerous insecticides. Additionally, the insecticide is applied more frequently and at greater concentrations (Pimental and Burgess, 1985). However, this does not solve the problem and often result in a switch to new insecticides which are usually more expensive than the previous ones. A consequence of these increases in dosages and frequencies of

application, as well as the changes to new and invariably more expensive compounds, is the direct increase in cost of pest control.

Development of resistance

Insecticide resistance develops due to genetic variation in the insect populations. A few individuals in the original target insect population survive a given dose of an insecticide. Generally, unaffected (resistant) individuals differ from affected (susceptible) individuals either in the nature of the insecticide's target site in the insect, or in the manner in which the insect breaks down the toxin molecules (Michaud, 1997). When the insecticide is applied, individuals who survive are able to pass their genes on to the following generations. Over time, a greater proportion of the insect population is unaffected by the insecticide. That is, resistance to insecticides develops by the selection of individuals that have the genes conferring resistance and by elimination of individuals without such genes; i.e., natural selection.

Mechanisms of insecticide resistance

Insects use different resistance mechanisms these include: behavioral, physiological, and biochemical resistance, as well as resistance due to target site insensitivity. Generally, when insects come into contact with an insecticide, the initial resistance mechanism may be reduced penetration of the insecticide through the cuticle. If the insecticide enters the organism, modifications of target sites or metabolism may result in target-site insensitivity or detoxification, respectively (Scott, 1990; Soderlund and Bloomquist, 1980). In some resistant insects, insecticides may be excreted from the organism at a greater rate (Matthews, 1980). Insects may also develop behavioral resistance by avoiding insecticides, (Scott, 1990; Mallet, 1989). Most of these

mechanisms may entail processes which are energy demanding; for example, enzyme production and modification of structures. Therefore insects exhibiting such mechanisms may express some kind of energy or fitness cost.

Behavioral resistance mechanism

Behavioral resistance results from actions evolved in response to selective pressure exerted by the toxicant (Lockwood et al., 1984). These actions enhance the ability of a population of insects to avoid the lethal effects of that toxicant. For example in mosquito, *Anopheles gambiae,* an indoor strain is susceptible to DDT sprays applied on walls inside homes however, another strain has developed behavior such as not adhering to treated walls and staying outdoor and therefore is not susceptible to DDT. It has become dominant because its behavior allowed it to avoid exposurs to the insecticide. Behavioral resistance to insecticides has also been documented in other insects including German cockroach (Ross 1992), the horn fly, *Haematobia irritans* (L.) (Diptera: Muscidae) (Zyzak et al., 1996), and the saw toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Cucujidae) (Watson and Barson, 1996). Behavioral resistance has been genetically analyzed (Mallet, 1989; Wood, 1981; Gould, 1984; Lockwood, 1984) which demonstrates that this mechanism of resistance can also be subject to selection.

Physiological resistance mechanisms

Physiological resistance involves: decreased penetration, increased excretion, and sequestration of insecticide. It is any form of resistance that reduces toxicity through changes in basic physiology. The insect tolerates the chemical by altering one or more physiological functions.

Resistance due to decreased penetration

The role of reduced penetration of insecticides through the insect cuticle was first reported as a resistance mechanism in the 1960's (Scott, 1990; Forgash et al., 1962). Decreased cuticular penetration could result from modification of the structure or composition of the cuticle such as, additional waxy layers in resistant strains. This mechanism typically confers low levels of resistance across a range of insecticides (Scott, 1990), but it can also enhance resistance levels when acting in conjunction with other mechanisms (Plapp, 1986; Plapp and Hoyer, 1968). Decreased penetration has been reported in some resistant insects, such as, the house fly (Plapp and Hoyer, 1968, Wen and Scot, 1999) and the German cockroach (Valles et al., 2000).

Resistance due to accelerated excretion

Accelerated excretion is one of the mechanisms involved in insecticide resistance. Using this mechanism, the concentration of the toxicant at the target site never reaches a threshold to be effective. For example, rate of excretion in malathion resistant lesser grain borers, *Rhizopertha dominica* (Fabricius) (Coleoptera: Bostrichidae), is faster than in susceptible strain (Matthews, 1980). In the Western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae), carbaryl resistant strains had an increased rate of excretion compared with the susceptible strain (Scarf et al., 1999). Rate of excretion was greater in diazion resistant flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), than the susceptible strain (Zhao et al., 1994). Rapid excretion was also observed as a mechanism of pyrethroid resistance in the tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) (Ottea et al., 1995).

Resistance due to sequestration/storage

Resistance due to sequestration involves storage of the toxicant at sites other than the target site. For example resistant peach potato aphid, *Myzus persicae* (Sulzerz), produces very large quantities of carboxylesterase E4 (the enzyme that catalyzes the hydrolysis of organophosphates and carbamates in the aphid), about 3% of the total protein in resistant aphids. However, resistant aphids showed low catalytic activity toward insecticide. It has therefore been suggested that the resistant effect caused by E4 is not mediated by hydrolysis but by storage/sequestration (Devonshire and Moores, 1982). Resistance due to sequestration has been reported in the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Gunning et al., 1996), Colorado potato beetle, *Leptinotarsar decemlineata* (Say) (Coleoptera: Chrysomelidae) (Lee and Clark, 1996), and the German cockroach (Prabhakaran and Kamble, 1995).

Biochemical resistance mechanisms

Biochemical resistance mechanisms include: resistance due to metabolic detoxification, oxidase-mediated metabolic detoxification, metabolism by Glutathione S-Transferases, and hydrolases-mediated metabolic detoxification. Biochemical resistance mechanisms are those that change the chemical structure of the toxin and render it nonfunctional.

Resistance due to metabolic detoxification

Insects have evolved with their hosts and to survive they possess enzymes to protect themselves from xenobiotic compounds produced by their hosts and from the environment. Metabolic detoxification is generally a two-step process. In phase one, the structure of the toxin is chemically altered with enzymes (oxidases, hydrolases, or

glutathione S-transferase) while in phase-two the altered toxin is converted into more water soluble metabolites which can easily be excreted.

Oxidase-mediated metabolic detoxification

Cytochrome P450 (P450s) comprise a large gene superfamily. In insects, the most common oxidases are P450 monooxygenases (Scott, 1991) found in the endoplasmic reticulum. These enzymes are normally involved in synthesis and degradation of hormones and pheromones (Agosin, 1985; Reed et al., 1994; Anderson et al., 1997) and detoxification of pesticides (Agosin, 1985, Berge et al., 1998) and plant toxins (Berenbaum and Feeny, 1981, Berenbaum, 1991, Harrison et al., 2001). The large variety of substrates metabolized by monooxygenase is due to the presence of multiple P450 isoforms in each species (e.g., 86 isoforms in the fruit fly, *Drosophila melanogaster* (Meiger) (Diptera: Drosophilidae) (Adams et al., 2000), in addition, each P450 may have several substrates (Rendic and Di Carlo, 1997). Insects commonly develop insecticide resistance by increasing monooxygenase activity leading to enhance detoxification of insecticides. Resistant insects induce or constitutively over express P450 isoforms. Examples include the diazinon resistant Rutgers housefly (Carino et al., 1994), malathion resistant fruit fly (Waters et al., 1992), and the pyrethroid resistant housefly (Liu and Scott, 1998) all of which over express P450s.

Metabolism by Glutathione S-Transferases

Glutathione S-Transferase (GST) usually involves conjugation of xenobiotics with endogenous substrates such as glutathione (Dauterman, 1983). GST works to convert a reactive lipophilic molecule into a water soluble conjugate so that it becomes easy to excrete (Habig et al., 1974). Fukami and Shishido (1966) were the first to

discover that organophosphorus insecticides could be degraded by GST. Later, Lewis (1969) and Lewis and Sawicki (1971) found that an organophosphate resistant housefly strain had a high glutathione-dependent diazinon and parathion degradation rate. Since then, there have been many reports of high levels of degradation in resistant insects compared with susceptible strains (Clark and Dauterman, 1982; Dauterman, 1986, Rufingier et al., 1999; Kostaropoulos et al., 2001).

Hydrolases-mediated metabolic detoxification

Hydrolases are present in most organisms and play a significant role in the metabolism of oganophosphates and pyrethroids insecticides (Dauterman, 1986). Higher hydrolase activity associated with organophosphate resistance has been reported in other organophosphate resistant insects such as the tobacco budworm, (Fabricius) (Goh et al., 1995).

Resistance due to target site insensitivity

The best known examples of target site insensitivity are altered acetycholine and resistance due to insensitivity of sodium channel (*kdr*). The first affects carbamate and organophosphate insecticides while the latter affects DDT and pyrethroid insecticides.

Altered acetycholinesterase

Organophosphate and carbamate insecticides exert their neurotoxic effects by inhibiting the enzyme acetycholinesterse (AchE). AchE cleaves the neurotransmitter acetycholine after it crosses the synapse converting it to choline and acetic acid. The insecticides bind to the active site of AchE thus inhibiting the activity of AchE, leading to continuous stimulation of the postsynaptic neuron followed by failure of the nervous system and death. The enzyme is altered such that the insecticde can no longer bind.

Altered AchE has been reported in S. exigua by Bryne and Toscano (2001), and Zhu and Clark (1995).

Resistance due to insensitivity of sodium channel

Pyrethroids and DDT act on the insect nervous system by slowing the action potential. They initiate repetive discharge in motor and sensory axons and convulsive activity, which eventually causes paralysis and death. Physically altered sodium channels result from mutations at target site and confer knockdown resistance (*kdr*) to DDT and pyrethroids. Knockdown resistance was first observed in DDT resistant adult houseflies (Busvine, 1951). Kdr also confers cross resistance. Kdr mechanism has been reported in several insects including housefly as early as the 1960's (Plapp and Hoyer, 1968) and the German cockroach.

The pests

Two insect pests were used in this study: the beet armyworm, Spodoptera exigua (Hübner) (Lepidoptera: Noctuidae) and the German cockroach, *Blattella germanica* (Linnaeus) (Dictyoptera: Blattellidae). The beet army worm is an agricultural pest and control is achieved by the use of *Bacillus thuringiensis* (Berliner) (Bt) whereas, the German cockroach is a household pest that is often controlled with pyrethroid insecticides. The mode of action of Bt and pyrethroids and the mechanisms of insecticide resistance in *S. exigua* and *B. germinica* differ.

Beet armyworm as an insect pest

The beet armyworm is a widely distributed polyphagous pest of numerous cultivated crops including cotton, tomatoes, cerelery, lettuce, cabbage, and alfafa. It is usually considered a secondary pest as populations generally build after natural enemy

populations have been reduced from application of broad spectrum insecticides (Ruberson et al., 1994; Graham et al., 1995). As a lepidopterian, *S. exigua* under goes complete metamorphosis. Eggs are greenish to white in color and are laid in clusters of 50 to 150 eggs per mass on the lower surface of a leaf. Depending on temperature, eggs hatch in two to three days into larvae that usually develop through five instars. First and second instar larvae are pale green to yellow in color and later acquire pale stripes during third instar. Larvae become darker dorsally with a dark lateral stripe as fourth and fifth instars. Pupation occurs in the soil and the pupae are light brown in color measuring 15 to 20 mm in length. The duration of the pupal stage is about six to seven days during warm weather. The adult moths are moderately sized with a wing span of 25 to 30 mm.

The beet armyworm, migrates long distances to find suitable habitats (Mikkola, 1970). It is able to overwinter as pupae although it does not have a diapause mechanism (Fey and Carranza, 1973). Its polyphagus feeding habit allows it to keep growing on weeds in crop field during winter, although cold temperatures slow its life cycle (Fey and Carranza, 1973; Kim and Kim, 1997).

Larvae feed on both foliage and fruit causing serious damage. Due to its polyphagous nature, *S. exigua* has a long history of exposure to a broad array of insecticides. Not surprisingly, it has developed physiological resistance to most classes of insecticides, including chlorinated hydrocarbons, organophosphates, carbamates (Meinke and Ware 1978), cyclodienes (Cobb and Bass, 1975), and pyrethroids (Brewer and Trumble, 1989). However, the use of less toxic alternatives to conventional insecticides, including biological control and botanical extracts (Berdegué et al., 1997) can reduce beet armyworm populations and larval damage to crop plants. However, the

most successful control using less toxic methods has been achieved with *B. thuringiensis* formulations. There is concern about widespread development of Bt resistance in *S. exigua* because field resistance to several Bt toxins has already been documented in several lepidopteran families (Tabashnik, 1994) and the introduction of transgenic plants expressing single Bt proteins could further increase selection pressure.

Mode of action of Bacillus thuringiensis and mechanism of resistance to S. exigua

B. thuringiensis is a gram-positive, spore-forming soil bacterium. When a susceptible insect ingests Bt toxin, the proteins are solubilized in the midgut and become proteolytically activated by midgut proteases. Activated toxin binds to specific receptors on the midgut epithelial membrane (Hofmann *et al.,* 1988; Van Rie *et al.,* 1989, 1990). Membrane bound toxins form pores in the cell membrane that result in osmotic lysis of the mid-gut epithelial cells and insect death (Knowles and Ellar, 1987).

The best-known mechanism of resistance in *S. exigua* is that of reduced binding. Moar *et al.* (1995) reported that binding experiments with *S. exigua* indicated that, binding of Cry1C toxin was reduced in Cry1C resistant insects compared with susceptible insects. However, other resistance mechanisms, including reduced binding have been reported in other Bt resistant insects for example, in *P. xylostella* reduced binding is the only known resistance mechanism (Ferre et al., 1991). Another resistance mechanism is the absence of gut proteinase. In addition to reduce binding in the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Van Rie et al., 1990b), resistance is linked to the absence of a major gut proteinase. Presumably, this proteinase is associated with the proteolytic cleavage and activation of Bt protoxins (Oppert et al., 1994). Reduced toxin binding and decreased activation of toxin, are thought to occur in

tobacco budworm, *H. virescens* (Michaud, 1997). Behavioral detection and avoidance of both formulated and purified Bt toxins have been reported in the larvae of Colorado potato beetle, *L. decemlineata* feeding on transgenic Bt potato, resistant larvae avoided feeding on high dose Bt plants (Hoy and Head, 1995).

German cockroach as an insect pest

The German cockroach has a world wide distribution and is the most common insect pest around homes, apartments, and restuarants. German cockroaches are found primarily in kitchens, bathrooms, or other areas with high levels of moisture and humidity (Robinson, 1996). The German cockroach undergoes paurometamorphosis. Females carry their egg cases or oothecae until they are ready to hatch. In each egg case, there are 18 to 50 eggs. Eggs hatch into nymphs that develop through approximately seven instars. Nymphs have two longitudinal streaks on the back and develop into adults that are ~16mm in length, tan in color with two longitudinal streaks on the pronotum (Benson and Zungoli, 1982; Robinson, 1996). The male is light brown and has a slender abdomen while the female is slightly darker in color and has a broader abdomen. German cockroaches contaminate food and produce an unpleasant odor (Ebeling, 1975). Furthermore, they are a threat to human health transmitting pathogens, such as viruses, helminthes, protozoa, fungi, and bacteria (Brenner, 1995). German cockroaches are also a source of allergens, and within the last decade, cockroach-induced asthma has been recognized as a serious health problem (Roberts, 1996).

Much research has been directed toward the German cockroach because of its potential as a vector of human disease causing pathogens. Non-chemical methods such as physical, mechanical, and biological control have been employed for control however,

chemical control, particularly using insecticidal baits, has been very efficient and successful (Benson, 1993; Benson and Zungoli, 1982). Unfortunately, the major impediment to effective control with conventional insecticides is the development of high levels of resistance. German cockroaches have developed resistance to all traditional insecticides used for its control (WHO, 1992).

Mode of action of Pyrethroids and mechanisms of resistance to B. germanica

Due to development of resistance by German cockroaches to all other insecticides, pyrethroids were introduced to replace the resistant-prone and environmentally unsuitable organochlorines, cyclodienes, and organophosphates. Pyrethroids possess high activity and can be applied at extremely low doses to achieve effective control. Furthermore, they are safe to mammals, have low environmental impact, and are immobile in the soil (Elliott, 1989).

The principal site of action of pyrethroids is the voltage-gated sodium channel of nerve cells (Soderlund and Bloomquist, 1989; Narahashi, 1992). These insecticides alter the gating kinetics of the sodium channel so that the open time of the channel is increased after the passage of the depolarizing pulse of an action potential. This action causes the repetitive firing of neurons and results in over stimulation of the insect and death.

The mechanisms involved in pyrethroid resistance include: decresed cuticular penetration, behavioral avoidance, and metabolic detoxification. Decreased rate of cuticular penetration has been reported as one of pyrethroid resistant mechanisms in German cockroaches (Valles et al., 2000; Wu et al., 1998), but see Wei et al. (2001). Wei et al. (2001) did not find decreased cuticular penetration as a resistance mechanism, suggesting that the mechanisms of pyrethoid resistance vary among German cockroach

strains. Behavioral avoidance has also been reported in the German cockroach (Ross, 1992). Resistance due to metabolic detoxification by P450 monooxygenases (Wu et al., 1998; Valles et al., 2000; Wei et al., 2001), and hydrolases (Scott, 1990; Wu et al., 1998; Valles et al., 2000; Wei et al., 2001) have been reported.

Fitness of insecticide resistant insects

The fittness of an organism is measured by the number of offspring it is able to produce in the next generation. In a population, alleles conferring resistance are usually rare before the population is exposed to a pesticide, but increase as the susceptibility of the population declines (Hollingsworth et al., 1997). These alleles have been presumed to exert negative effects on fitness in the absence of the pesticides (Hoffman and Parsons, 1991) although fitness costs can vary considerably (Georghiou and Taylor, 1977; Roush and McKenzie, 1987; Roush and Daly, 1990; Denholm and Rowland, 1992; Tabashnik, 1994). Resistance has been reported to decline in absence of insecticide (Tabasnik et al., 1994; Rahardja and Whalon, 1995) and in Bt this reversion may be associated with increase fitness (Tabasnik, 1994). Reduced fitness is usually associated with resistance genes. The pleiotropic effects associated with resistance alleles in insects can be seen as reductions in pupal mass, survival, fecundity, egg hatch, developmental time, and mating success (McDonald et al., 1987; Daly and Fitt, 1990; Brewer and Trumble, 1991; Ffrenchconstant et al., 1993; Roush, 1994; Idris and Grafius, 1996; Hollingsworth et al., 1997; Han et al., 1999; Oppert *et al.,* 2000; Liu *et al.,* 2001).

The fitness costs of insecticide resistance can be expressed as phenotypic characters such as reduced mass and egg hatch, however, other fitness component may exist which may contribute to these observed effects. For instance, most of the

insecticide resistant mechanisms such as detoxification are energy demanding which may result in increased metabolic rate. It has been reported that detoxifying enzyme production increases only in response to exposure to pesticide (Terriere, 1983). Therefore, increased metabolic rate may indicate reduced fitness because more energy may be used to detoxify insecticide than for growth and development. In addition, increased metabolic rate would result in increased respiratory water loss. One method to determine if there is a metabolic cost of insecticide resistance is to measure the metabolic rates; specifically, CO_2 production and O_2 consumption.

Metabolic Rates

Metabolic rate, defined as a measure of the total energy metabolized by an animal in unit time, is a commonly measured physiological variable. However, this measurement is usually affected by activity. For instance, a flying insect has a metabolic rate many times greater than that of a resting individual. To provide standards for comparison, several categories of metabolic rate have been recognized. These include standard metabolic rate (SMR) defined as metabolic rate of ectotherms during rest with no spontaneous activity, no digestion of food, and no physical, thermal, or psychological stress (Willmer et al., 2000). Standard metabolic rates can be obtained at a specific temperature from ectothermic animals such as the Beet armyworm and the German cockroach, whose metabolic rates are strongly influenced by temperature. Active metabolic rate refers to metabolic rate during activity. In this study, SMR metabolic rates of the Beet armyworm and the German cockroach were measured as carbon dioxide ($\dot{V}_{CO_2}$) production and oxygen consumption ($\dot{V}_{O_2}$) using close system respirometry.

A major constraint when using metabolic rate for inter and intra-species comparison is that metabolic rates of organisms follow an allometric rather than an isometric (1:1), relationship with body mass. Fortunately, this can be resolved by mass scaling. For example using Huxley's simple allometric equation $MR = aM^b$ (where MR is metabolic rate; a is a proportionality coefficient, M is body mass; and b is the mass exponent), the basal metabolic rate for birds and mammals increases proportionally to body mass according to a power of 0.70-0.75 and 0.62-0.67 for insects (Willmer, 2000). Therefore, in this study, our results are reported in mass-specific terms as rates of carbon dioxide production (ml CO_2 g^{-1} h^{-1}) and oxygen consumption (ml O_2 g^{-1} h^{-1}).

From measurements of O_2 consumption and CO_2 production rates, respiratory quotient (RQ) can be obtained. RQ is defined as the molar ratio of carbon dioxide produced and oxygen consumed per unit time during the metabolism of a foodstuff (Schmidt-Nielsen, 1990). From RQ values it is possible to estimate what substrate an organism is metabolizing because different substrates require different amounts of O_2 for metabolism and result in different amounts of CO_2 produced. RQ values of 1.0, 0.72, and 0.84 indicate carbohydrate, fat, and protein metabolism, respectively (Bartholomew, 1977). RQ values outside this range (0.7-1.0) are rarely encountered however, RQs below 0.7 could be associated with gluconeogenesis while increase above 1.0 could result from the conversion of carbohydrate to fat (Cantarow and Schepartz, 1968).

Temperature is an abiotic factor that strongly affects the metabolic rate of ectothermic organisms. Increases in temperature generally results in increases in metabolic rate. It is well known that for every 10^{o}C rise in temperature, metabolic rate doubles this is known as the Q_{10} effect (Wilmer et al., 2000). However, since most

physiological processes such as metabolic rate are regulated by enzymes which are denatured by high temperature, death may result if the temperature is above a certain threshold.

Discontinuous gas exchange (DGC)

Insects can respire using either continuous or discontinuous gas exchange patterns. One of the most striking types of gas exchange pattern in insects is a discontinuous pattern. The discontinuous gas exchange cycle (DGC) first described in detail in lepidopteran pupae (Levy and Schneiderman, 1966), has been reported in several insect and non-insect orders including blood-sucking bugs (Punt, 1950), cockroaches (Kestler, 1991), beetles (Lighton, 1988,), grasshoppers (Hadley and Quinlan, 1993), ants (Lighton and Berrigan, 1995), mites (Lighton and Duncan, 1995), ticks (Lighton et al., 1993), termites (Shelton and Appel, 2001), and solphugids (Lighton and Fielden, 1996). The DGC has been defined as the uncoupling of CO_2 release and O_2 uptake through the spiracles (Lighton, 1994). A typical insect DGC is divided into three phases: closed, flutter, and open that can be described as the activity of the spiracles and the pattern of gas exchange. During the closed phase (C), the spiracles are closed, resulting in little or no gas exchange. The insect consumes O_2 from its endotracheal stores and cellular respiration produces CO_2 which is buffered by bicarbonate and proteins in the hemolymph. The result is a rise in the PCO_2 of the hemolymph and tracheae, and a decline in PO_2 in the endotracheal space. In most insects, the closed phase is followed by the flutter phase (F). Due to accumulation of carbon dioxide in the hemolymph, a fall in endotracheal pressure occurs. The spiracles rapidly flutter open and closed. This leads to bulk movement of air from the surrounding environment into the tracheal system; oxygen

enters the tracheal system at a rate sufficient to meet tissue respiration requirements while a small amount of carbon dioxide escapes. Finally, because of rising hemolymph PCO_2, the spiracles open, and the accumulated CO_2 is released (open phase). Then the spiracles close again, initiating a new closed phase. The closed phase is marked by very low gas exchange rates and nearly no respiratory water loss. On the other-hand, the flutter phase shows a small increase in carbon dioxide emission rate and a much larger increase in oxygen consumption rate with very low water loss while the open phase has maximum water loss (Lighton, 1996).

Adaptive significance of DGC

One theory holds that the DGC originated primarily as a means of reducing respiratory water loss (Kestler, 1985; Slama, 1988; Lighton, 1994) because water loss from the tracheal system would be nearly zero during the closed phase, and much reduced if the flutter phase is solely or predominantly convective, as has been demonstrated in moth pupae, cockroaches, and some ants (Levy and Schneiderman, 1966; Kestler, 1985; Lighton et al., 1993). This theory is supported by work of Williams et al. (1998) and Duncan et al. (2002). However, this theory has been questioned because when insects that can DGC are subject to water stress they do not DGC (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993; Lighton, 1994; Lighton and Garrigan, 1995; Lighton, 1996; Chown and Holter, 2000). Lighton and Berrigan (1995) proposed that the DGC may originally have evolved to enhance gas exchange efficacy under hypoxic and hypercapnic circumstances, without the excess water loss associated with continuous gas exchange (Lighton, 1994; 1996; 1998). Hypercapnia and hypoxia may limit diffusion of respiratory gases by reducing the concentration gradient of CO_2 and O_2 between intra-

tracheal space and the environment. By keeping the spiracles periodically closed, the concentration of O_2 and CO_2 decrease and increase in the intra-tracheal space, respectively, to levels lower/greater than O_2/CO_2 concentration in the environment. Thus gas exchange can take place due to concentration gradient when the spiracles are open (Lighton, 1998). More recently, Hetz & Bradley (2005) proposed a third possible explanation suggesting that insects breathe discontinuously when at rest to avoid oxygen toxicity. Their hypothesis is based on the fact that exposure of tissues to high levels of O_2 is unusual among biological systems and that high levels of O_2 can cause oxidative damage. Consequently, it is O_2 regulation that produces the observed discontinuous release of CO_2.

Effects of stressors on metabolic rate and gas exchange pattern (DGC)

Insects are subjected to environmental stressors such as insecticide and temperature extremes these may have drastic consequences on their respiration patterns and metabolic rate. Response to stressors (e.g., heat stress, cold stress, toxins/insecticidal stress, and metabolic stress) by insects (Rauschenbach *et al.,* 1987; Cymborovski, 1988; Ivanovic, 1991; Imasheva *et al.,* 1997; Harshman *et al.,* 1999) is of great interest because it influences survival and fitness (Djawdan *et al.,* 1997). The effects of stress on metabolic processes have been documented in insects (Djawdan *et al.,* 1997; Giesel *et al.,* 1989; Krebs and Feder, 1998). Selection for stress resistance will lead to lower SMR and during stressful conditions, stress resistant organisms will have a lower metabolic rate (Hoffman and Parson, 1989). This has been observed in *D. melanogaster* by selecting for desiccation resistance (Hoffman and Parson, 1989; Djawdan *et al.,* 1997).

Increasing temperatures may alter gas exchange from discontinuous to continuous (Punt, 1950; Lighton, 1990). At lower temperatures, metabolism is low and CO_2 production is reduced thus the spiracles remain closed and are only opened when the amount of CO_2 within the trachea has increased to a certain threshold. However, at higher temperatures, with increased metabolism more CO_2 is released in the tissue per unit time causing the spiracles to flutter continuously to eliminate carbon dioxide most rapidly.

In the American cockroach, *Periplaneta americana* (L.) (Dictyoptera: Blattidae), sublethal and lethal doses of insecticides led to alteration in respiration pattern; from discontinuous gas exchange cycle (DGC) to continuous or cyclic (Kestler, 1991). Changes in respiration patterns may be due to autointoxication (Pipa and Delcomyn, 1982), result of release of neurohormones (Slama and Miller, 1987) or a change in the CO_2/O_2 threshold. Also, changes in gas exchange patterns could result from dehydration because when the spiracles are open continuously, more water is lost which will eventually result in death. A sublethal dose of an insecticide may increase total body water loss and result in an increase in searching for food/water. If for example, a liquid bait is applied when insects are water-stressed, effective control may be achieved more rapidly because more active ingredient will be consumed.

Effects of developmental age on metabolic rate

Change in energy flow during development of the red flour beetle, Tibolium castaneum, (Coleoptera: Tenebrionidae), has been reported (Klekowski and Duncan, 1975; Prus, 1975). The relationship of metabolic rate and pupal age is characteristically U-shaped for many holometabolous insects (Prosser, 1973) indicating a change in

metabolic rate over developmental time. This is so because insect growth occurs in a cyclic fashion, with periods of active growth alternating with relatively passive or non-active stages. It involves series of molts during which there may be considerable cellular breakdown and resynthesis. It is therefore important that study of insect metabolism and evaluation of the adaptive significance of gas exchange patterns should take developmental time into consideration. However, most work on metabolism and DGC using flow though respirometry has not taken this into consideration. This study will be the first to report such findings using DGC.

Goals and objectives

In Chapter II, the effects of *Bacillus thuringiensis* Cry1C toxin on the metabolic rate of Cry1C resistant and susceptible *S. exigua* were measured. The primary objective of this study was to determine whether there is a metabolic cost involved in resistance. However, RQ values were also measured to determine what food substrates were being metabolized. To achieve these goals, a susceptible (SeA) and resistant (Cry-) strains of *S. exigua* were used. Experimental insects consisted of: a susceptible strain (SeA) that had been reared in laboratory for several years without exposure to insecticide; a resistant strain reared on artificial diet (CryReg); a resistant strain reared on artificial diet containing 320µg/g toxin for five days (Cry5dT); and a resistant strain reared continuously on 320µg/g toxin (CryonT). Third and fifth instar larvae and 1 to 7 day old pupae of *S. exigua* were used. Closed system respirometry was used to measure metabolic rate because this technique allows for more accurate measurement of O_2 consumption.

In Chapter III, the goal was to test whether *Bacillus thuringiensis* Cry1C toxin, age, and temperature affect the discontinuous gas exchange patterns of *S. exigua* pupae. Flow through respirometry was used to measure CO_2 production patterns in real time. Because of the difference in sensitivity between CO_2 and O_2 analyzers only CO_2 production can be measured. We used 1 to 7 day old pupae at temperatures of 10, 15, 20, and 25^{o}C. Characteristics of the DGC (closed, flutter, and open phases) were compared between resistant and susceptible strains of *S. exigua.*

In Chapter IV, the effects of temperature (10-40^{o}C) on the metabolic rate of *B. germanica* was investigated using closed system respirometry. Three strains of German cockroach were used in this study: a susceptible strain ACY (American Cyanamid Co., Clifton, NY) maintained in the laboratory without exposure to insecticide for >30 years, a black body mutant strain that has also been maintained in the laboratory for >30 years, and an insecticide resistant strain (Apyr-R) collected from infested apartments in Opelika, Lee County, AL, U.S.A after control failure with pyrethroid insecticides.

In Chapter V, the goal was to test whether insecticide and temperature affect the discontinuous gas exchange patterns of pyrethroid resistant and susceptible *B. germanica*. Flow through respirometry was used to measure CO_2 production patterns. We tested adults German cockroaches at temperatures of 10, 15, 20, 25, 30, and 35^{o}C. Characteristics of the DGC (closed, flutter, and open phases) were compared between the resistant and susceptible strains. The rate of water lose during a DGC was compared between both strains.

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CHAPTER 2: EFFECTS OF *BACILLUS THURINGIENSIS* CRY1C TOXIN ON THE METABOLIC RATE OF CRY1C RESISTANT AND SUSCEPTIBLE *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE)

INTRODUCTION

Bacillus thuringiensis (Berliner) (Bt) is a gram-positive, spore-forming bacterium that has become the most promising alternative to the use of chemical insecticides for management of insect pests (Whiteley and Schnepf, 1986). When a susceptible insect ingests Bt toxin, the proteins are solubilized in the midgut and become activated by midgut proteases. Activated toxin binds to specific receptors on the midgut epithelial membrane (Hoffmann *et al.,* 1988; Van Rie *et al.,* 1989, 1990). Membrane bound toxins form pores that result in osmotic lysis of the midgut epithelial cells and insect death (Knowles and Ellar, 1987).

Evolution of resistance to Bt toxins by insects is a serious threat to the efficacy of Bt as an insecticide and toxin-producing transgenic plants, which are generally pest specific and environmentally safe. Mechanisms of Bt resistance could be associated with one or several of the steps of activation and toxic action of Bt Cry proteins (Ferré and Van Rie, 2002). Reversal of resistance to Bt Cry1A(c) has been reported in resistant

diamondback moth, *Plutella xylostella*; restoration of binding was observed after exposure to the toxin was discontinued (Tabashnik *et al.,* 1994).

Although insects have developed physiological resistance mechanisms to most pesticides, costs may be associated with these mechanisms. For example, pyrethroid resistant tobacco budworms, *Heliothis virescens*, produced significantly fewer eggs (Plapp *et al.,* 1990) and dieldrin resistant *Anopheles* spp. mosquitoes spend less time searching for hosts (Rowland, 1987). In *P. xylostella*, resistance to Bt was correlated with lower egg hatch, reduced survival, reduced mating success of males and reduced fecundity (Groeters *et al.,* 1993; 1994). Carriere *et al.* (2001) also showed that there were over wintering fitness costs involved with resistance to Cry1Ac in *Pectinophora gossypiella*.

Response to stress (e.g., heat stress, cold stress, toxins/insecticidal stress and metabolic stress) by insects (Rauschenbach *et al.,* 1987; Cymborovski, 1988; Ivanovic, 1991; Imasheva *et al.,* 1997; Harshman *et al.,* 1999) is of great interest because it influences survival and thus helps determine fitness (Djawdan *et al.,* 1997). Some effects of stress on metabolic processes have been documented in insects (Djawdan *et al.,* 1997; Giesel *et al.,* 1989; Krebs and Feder, 1998). It has been suggested that selection for stress resistance will result in lower standard metabolic rates and that organisms with increased stress resistance will have a lower metabolic rate during stressful conditions. For example, these effects have been observed experimentally in *Drosophila melanogaster* after selection for desiccation resistance (Hoffman and Parsons, 1989; Djawdan *et al.,* 1997).

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a cosmopolitan pest that attacks plant species including important crops such as cotton, cabbage, maize, tomatoes, soybeans and cauliflower. *Spodoptera exigua* has developed broad physiological resistance to a variety of toxicants including pyrethroids (Brewer and Trumble, 1989) and cyclodienes (Cobb and Bass, 1975). Control has been successful with the use of Bt formulations, however, there is concern about widespread development of Bt resistance in *S. exigua* because field resistance to Bt toxins has been documented in several Lepidopteran families (Tabashnik, 1994) and the introduction of transgenic plants expressing single Bt proteins could further increase selection pressure. A high level of Cry1C resistance (>500-fold) was developed in *S. exigua* after laboratory selection (Moar *et al.,* 1995). Binding experiments with *S. exigua* indicated that, although binding of Cry1C toxin was reduced in Cry1C resistant insects compared with susceptible insects, reduced binding was probably not the major mechanism of resistance (Moar *et al.,* 1995). Other possible mechanisms of resistance could include altered proteolytic processing, reduced protease titres necessary to further cleave activated toxin (Oppert *et al.,* 1997; W. Moar, personal observation), reduced binding due to decreased or modification of toxin binding sites or detoxification (Ferré and Van Rie, 2002). Energetic costs may be associated with Bt resistance. For example, pesticide detoxification involves enzymatic breakdown of ingested or absorbed toxins (Soderland and Bloomquist, 1990), a process that may increase energy use as detoxification enzymes are synthesized. In addition, replacement of damaged midgut cells could also increase energy use.

Although extensive research has been conducted with Bt toxins against *S. exigua* (Moar *et al.,* 1986; Luo and Adang, 1994; Stapel *et al.,* 1998; Ashfaq *et al.,* 2000)

including the highly effective Cry1C toxin (Moar *et al.,* 1995; Berdegue *et al.,* 1996), no information is available concerning the effects of Bt toxins (Cry1C) on the metabolic rate of *S. exigua*. Such studies are necessary to help provide a link between the physiology and ecology of *S. exigua*, which will enable the development of better pest management strategies. Therefore, to determine whether an energetic or metabolic cost to Bt resistance exists, I measured metabolic rates (CO_2 production and O_2 consumption) of resistant and susceptible S. exigua.

MATERIALS AND METHODS

Insects

I used two strains of S. exigua for this study: a standard susceptible strain (SeA) and a resistant strain (Cry1C). Both colonies were established at Auburn University, Alabama, USA, with insects collected from cotton (Moar *et al.,* 1995). The susceptible colony was maintained on artificial diet (Chalfant, 1975) while the resistant colony was reared on artificial diet containing 320µg Cry1C toxin per g of diet for > 35 generations and was maintained on the 320µg Cry1C toxin diet for the duration of these experiments. Moar *et al.* (1995) reported a >500-fold resistance compared with the susceptible colony. Since 1995, the Cry1C resistant colony routinely exhibited negligible mortality at 320µg Cry1C toxin per g of diet, whereas the LC_{50} for the susceptible colony was usually between 5 and 20µg Cry1C toxin per g of diet (W. Moar personal observation). The exact LC_{50} for the resistant colony at the time of these experiments could not be determined because of limited availability of Cry1C toxin and the large concentrations required to establish the LC_{50} value with this population (Moar *et al.,* 1995).

Toxin and treatments

Cry1C from Bt was expressed in a Cry (-) strain of Bt, purified and trypsin-activated as previously described (Moar *et al.,* 1995). Toxin was incorporated into artificial diet by adding 6 ml toxin (5.5 mg toxin per ml 5 mM CAPs buffer, pH 10.5) and 14 ml distilled water to 80 g of diet and blended for 1-2 min. The mixture was poured into 35 ml plastic cups (Fill-Rite Corp. Newark, NJ, USA) and 10-20 neonates were loaded per cup. The same Cry1C toxin aliquot was used for both maintaining the colony and for the experiments described below.

Treatments included the SeA reared on artificial diet, the Cry1C resistant strain reared on artificial diet (CryReg), Cry1C resistant strain reared continuously on artificial diet containing 320µg Cry1C toxin per g of diet (CryonT) and the Cry1C-resistant strain reared on artificial diet containing 320µg Cry1C toxin per g of diet for 5 days after egg hatch then transferred to artificial diet without toxin (Cry5dT) (Table 2.1). CryonT and Cry5dT treatment groups allowed us to test the direct influence of toxin on metabolic rate. CryonT simulated a situation where larval infestation occurs in a field planted exclusively with transgenic crops (exposed to toxin during the entire larval period) while Cry5dT represents bioassay and laboratory rearing scenarios where genetically resistant insects are selected with toxin for 5-7 days. CryReg represents genetically resistant insects that have not been selected or induced with toxin as might occur in refugia or on an untreated host. The SeA group represents susceptible insects that have never been exposed to toxin. At even very low doses (~1 µg Cry1C /g diet) most SeA died as neonates and the few that survived never developed to the third stadium.

Insects were reared at 28 ± 2°C in a LD 16:8h photo cycle. The developmental time (length of the larval period) of SeA, CryReg and Cry5dT required about 2 weeks while that of CryonT was delayed by ~1.5 weeks (B. Dingha personal observation). Therefore, insect populations could not be initiated simultaneously as CryonT required additional time to reach desired instars and they were therefore initiated before SeA and CryReg. One day-old third and fifth instar larvae and 1-7-day old pupae were used for all the treatments. To ensure that only one day-old third and fifth instars were used, second and fourth instar larvae for each treatment group were selected randomly a day before they moulted into third or fifth instars. For pupae, prepupae of each treatment were selected randomly and the next day, newly eclosed 1 day-old pupae were removed and placed individually in a plastic cup containing artificial diet which provided moisture. The same pupae were used daily to measure oxygen consumption and carbon dioxide production during the 7 day experiment. A randomized complete block design was used for this study. The entire experiment was repeated twice (block) with each treatment consisting of 10 insects for a total of 30 insects per treatment. The sex of the pupae was not determined because preliminary studies indicated that there was no difference in the amount of oxygen consumed and carbon dioxide produced by male and female *S. exigua* pupae (B. Dingha, personal observation).

Respirometry

One day-old third and fifth instar larvae, and 1-7 day-old pupae from each treatment were weighed on an electronic balance to the nearest 0.01 mg and placed individually in respirometers constructed from either 1 or 5 ml syringes (Becton, Dickinson and Company, Ruthford, NJ, USA). Each respirometer had a small hole drilled

at the end of the gradation and a stopcock at the other (tip) end. Respirometers containing insects were connected to a manifold that provided dry, CO_2-free air at the rate of 100 ml/min and were flushed for ~10 min. Then the plunger was brought to the 0.8 ml (1 ml syringe) or 3 ml (5 ml syringe) gradation and the stopcock was adjusted to seal the respirometer. Respirometers were incubated at 25 ± 2 ^oC for 0.5-3 h depending on the syringe used and the size of the insect. Incubation time (from the moment the stopcock was closed until injection of the air sample) was recorded for each respirometer. We used video recordings of larvae in respirometers to detect movement. No difference in O_2 consumption or CO_2 production could be detected between completely still and minimally (<1 cm per recording at 25^oC) moving larvae (B. Dingha, personal observation).

Oxygen depletion and carbon dioxide enrichment in each respirometer was determined using a Sable Systems TR-3 respirometry system (Sable Systems, Henderson, NV, USA). Outside air was scrubbed of CO_2 and H_2O using a Whatman purge gas Generator (Whatman, Inc., Haverhill MA, USA), drawn through a computer-controlled base lining system, a Li–Cor (LI-6262; Li-Cor Inc., Lincoln, Nebraska, USA) CO_2 and H_2O analyser, a Sable Systems FC-1 Oxygen Analyser, and a Side-Track mass flow meter (Sierra Instruments Inc., Monterey, CA, USA) with a pump (GastMfg. Corp., Benton Harbor, MI, USA) at a rate of 100 ml/min at STP. The CO_2 analyser was calibrated with 94.9 ppm span gas (Air Products, Inc.) weekly. The oxygen analyser was internally zeroed and spanned to 20.94% O_2 daily. From the 1 ml and 5 ml syringes, 0.5 ml or 1 ml of air, respectively, was injected into a glass T- injector port with a replaceable rubber septum and crimp seal installed up stream of to the CO_2-H_2O analyser.

The gas sample passed through the system and data from the O_2 and CO_2 analysers were recorded using DATACAN V (Version 5.2; Sable Systems, Henderson, NV, USA) software. This procedure was performed repeatedly (daily) on all pupae for the entire 7 day experiment and for third and fifth instar larvae. Depletion (O_2) and enrichment (CO_2) volumes were integrated with respect to time. Rates of O_2 consumption and CO_2 production were calculated as the volume of O_2 and CO_2 (ml) divided by insect body mass (g), and incubation time (h) resulting in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in units of ml g^{-1} h^{-1}. Respiratory quotient (RQ) was calculated as the ratio of CO_2 produced ($\dot{V}_{CO_2}$) to O_2 consumed ($\dot{V}_{O_2}$). RQ values range from 1.00 for carbohydrate, 0.80 for protein and 0.71 for lipid metabolism (Bartholomew, 1977). Details on the calculations employed and general information on closed–system respirometry can be found in Lighton (1991b).

Statistical analysis: Mass scaling and RQ

Data were subjected to analysis of covariance (ANCOVA) to estimate the mass scaling coefficient between metabolic rate and body mass for each treatment group. Regression analysis was performed if there was a significant effect of mass to estimate the slope of the Log $\dot{V}_{O_2}$ or Log $\dot{V}_{CO_2}$ and Log mass relationship. A modified *T*-test ($P \leq 0.05$) (Sokal and Rohlf, 1981) was used to determine if the slopes were significantly different than 1. The slope of this relationship is the mass scaling coefficient and it was used to appropriately correct for the effect of body mass on $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ resulting in mass independent metabolic rate (MIMR). Mean ± SE RQ values for each treatment were compared with the standard value for lipid metabolism (0.71) using a modified *T*-test ($P \leq 0.05$) (Sokal and Rohlf, 1981).

Larvae

Analysis of variance (ANOVA) using the general linear model procedure (Proc GLM; SAS Institute, 1996) was used to examine $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, RQ and body mass. The GLM procedure was used to determine the effects of instar, strain and the instar by strain interaction on these variables. When the *F*-test for the effect was significant ($P<0.05$), least square means (LSMEANS; SAS Institute, 1996) were used to compare treatment means.

Pupae

Comparisons among treatments over time with regards to $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, RQ and body mass were analysed using repeated measures ANOVA. $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, RQ and body mass were measured daily for 7 days. Tests of fixed effects (PROC Mixed Procedure; Khattree and Naik, 1999; SAS Institute, 1996) were used to determine day, strain and day by strain interaction effects on the variables. Mean separations were carried out using least square means. A significance level of $P\leq0.05$ was used throughout.

RESULTS

Mass scaling

Larval third and fifth instars metabolic rate scaled allometrically with mass with a slope of 0.93 ± 0.043 (r^2=0.80, *F*=470, df=1, 118, *P*=0.0001). This value was not significantly different than 1, (*t*=0.19, df=118, $P\leq0.05$) so $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were mass corrected by dividing by body mass. However, the relationship of metabolic rate to body mass of pupae had a slope of 0.66 ± 0.054 (r^2=0.17, *F*=144, df=1, 693, *P*=0.0001) that

was significantly less than 1, (t=0.006, df=693, $P \leq 0.05$). Therefore, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ values were divided by body mass $^{0.66}$ to obtain MIMR.

Third and fifth instar larvae

Combining all treatment groups, third instar larvae had significantly greater metabolic rate ($\dot{V}_{O_2}$) than fifth instar larvae (Table 2. 2A). When both instars were combined, CryonT had significantly greater $\dot{V}_{O_2}$ than the other treatment groups (Table 2. 2A). For third instar larvae, $\dot{V}_{O_2}$ of CryonT (1.07 ± 0.05 ml $g^{-1}h^{-1}$) larvae was significantly greater than Cry5dT, CryReg and SeA larvae (~0.77 - 0.78 ml $g^{-1}h^{-1}$) (Fig. 2.1). However, there were no differences in metabolic rate ($\dot{V}_{O_2}$) among treatment groups for fifth instar larvae (Fig. 1). $\dot{V}_{CO_2}$ of third and fifth instars were significantly different when all treatment groups were combined (Table 2.2B). When both instars were combined, CryonT had significantly greater $\dot{V}_{CO_2}$ than the other treatment groups. For third instar treatment groups, $\dot{V}_{CO_2}$ of CryonT (0.6 ± 0.03 ml $g^{-1}h^{-1}$) larvae was significantly greater than all other treatments (~0.42 ml $g^{-1}h^{-1}$) (Fig. 2.2). However, there was no significant difference in metabolic rate ($\dot{V}_{CO_2}$) among treatment groups for fifth instar larvae (Fig. 2.2).

There was a significant (P<0.05) difference in body mass between instars and among treatment groups with the effect of treatment on body mass being significantly different among instars (Table 2.2C). CryonT had significantly reduced body masses for both third and fifth instar larvae when compared with other treatments (Fig. 2.3). RQ was not different between instars and treatment groups and was not influenced by the

instar by treatment interaction (Table 2.2D). Mean RQ for both instars and treatment groups was 0.54 ± 0.15 and was not significantly different (t= 0.44, df= 118, $P \leq 0.05$) from that of fat metabolism (0.71).

Pupae

Pupal metabolic rate ($\dot{V}_{O_2}$) varied significantly among days and treatment groups with a significant treatment group by day interaction (Table 2.3A). On the first pupal day, $\dot{V}_{O_2}$ of CryonT was significantly greater than all other treatment groups, which did not differ from one another (Fig. 2.4). Metabolic rate declined ~15-40% on day 2 in CryonT, SeA and Cry5dT compared with day one. The lowest metabolic rate ($\dot{V}_{O_2}$ of ~0.09 ml $g^{-1}h^{-1}$) of all treatment groups was observed on days 3 and 4; there was no significant difference among treatment groups (Fig. 2.4). $\dot{V}_{O_2}$ increased linearly during the remaining days of the pupal stadium until adult emergence (Fig. 2.4). From days 3-7 CryonT had the least metabolic rate even though this was not significantly different from the other treatment groups except on days 4 and 6 (Fig. 2.4). Table 2.3B shows a significant difference in the $\dot{V}_{CO_2}$ among days and treatment groups with no significant interaction between days and treatment groups. On the first pupal day, $\dot{V}_{CO_2}$ of CryonT was greater but not significantly different than all other treatment groups (Fig. 2.5). From days 2-7 significant or not, CryonT had the lowest $\dot{V}_{CO_2}$ (Fig. 2.5).

There was a significant difference in pupal mass among treatment groups throughout the 7 day experiment however; there was no treatment group by day interaction (Table 2.3C). CryonT pupae were significantly lighter, ~ 50% lighter, than

pupae in the other treatment groups (Fig. 2.6) consistent with the results obtained with third and fifth instar larvae (Fig. 2.3). Throughout the 7 day, CryReg weighed the most on each day followed by Cry5dT, SeA and CryonT for most days (Fig. 2.6). RQs were significantly different throughout the 7 day and among treatment groups (Table 2.3D). RQ's ranged between 0.43 ± 0.16 for CryonT at day 1 to 0.61 ± 0.14 at day 7 for CryonT. However none of the RQ's were significantly different (t=0.68, df= 24, P<0.05; t=0.26, df= 14, P<0.05, respectively) from 0.71 (lipid metabolism).

DISCUSSION

This study reports significant differences in metabolic rates among larval and pupal stages of Cry1C resistant and susceptible strains of *S. exigua* in response to exposure to Bt Cry1C toxin. Reduction in metabolic rate is a generalized response to stressors (e.g., toxins, insecticides, heat and cold) that could lead to an increased availability of resources for growth, reproduction and reduction in respiratory water loss (Chown and Gaston, 1999; Hoffmann and Parsons, 1989). Reduction in respiratory transpiration is of adaptive significance in water stressed *Drosophila* (Hoffmann and Parsons, 1989), ants (Lighton and Batholomew, 1988; Lighton, 1994, 1996), beetles (Lighton, 1991a) and a variety of other insects (Lighton, 1994, 1996; Wasserthal, 1996). However, my results do not support the generalized response to stress hypothesis. Metabolic rates of CryonT third instar larvae and 1 day-old pupae were the greatest and they weighed the least whereas, Cry5dT and CryReg had reduced metabolic rates and weighed the most. Therefore, in the case of third instar Cry5dT and CryReg, lower rather than higher metabolic rates are specific to short-term or no exposure to toxin. CryReg and Cry5dT may be able to produce detoxification enzymes that could lead to increased

metabolic rates, but probably require presence of toxin to induce production of detoxifying enzymes. In several studies, detoxifying enzyme production increased only in response to exposure to pesticide (Terriere, 1983). Therefore, increased metabolic rate could indicate reduced fitness because more energy may be used to detoxify Bt toxins or replaced damaged midgut cells than for growth and reproduction. In addition, increased metabolic rate could result in increased water loss, which may result in more rapid desiccation and eventually death (Duncan *et al*., 2002).

Generally, the $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ indicate that the resistant strain reared continuously on toxin (CryonT) had a greater metabolic rate compared with the other treatment groups. Also, masses of CryonT third and fifth instar larvae as well as pupae were significantly lower than the other treatment groups. Hostetler *et al.* (1994) reported no difference in the metabolic rate of organophosphate and pyrethroid insecticide resistant and susceptible strains of the German cockroach *Blattella germanica*. Differences in other fitness components such as pupal weight, survival, fecundity, egg hatch and mating success have been reported between Bt resistant and susceptible *P. xyllostella*, the Indian meal moth, *Plodia interpunctella*, and *P. gossypiella* (Groeters *et al.,* 1993; 1994; Oppert *et al.,* 2000; Liu *et al.,* 2001). Female size (mass) is positively correlated with fecundity and thus fitness (Tisdale and Sappington, 2001). Therefore, smaller larvae result in smaller pupae and presumably smaller, less fecund adults. Reduction in pupal mass and extended larval developmental time has been reported in *S. exigua* and Cry 1Ac-resistant *P. gossypiella* on transgenic cotton (Ashfaq *et al.,* 2000; Liu *et al.,* 2001). Perhaps the toxin interferes with nutrient uptake resulting in malnourished larvae and subsequent pupae. Another possible explanation for reduced body mass and increased $\dot{V}_{O_2}$ of the

CryonT treatment group is increased excretion. For example, chitin synthesis inhibitors are excreted more rapidly by *S. exigua* than by *S. littoralis* and *Leptinotarsa decemlineata*; increased rate of excretion was postulated to confer tolerance to these compounds (Van Laecke and Degheele, 1991).

The decrease in $\dot{V}_{O_2}$ of CryonT fifth instar larvae compared with third instars may have resulted from hormonal changes during metamorphosis; ecdysteroid titres increase in fifth instars suppressing larval genes and activating pupal genes necessary for metamorphosis (Nijhout, 1994). The similarity in metabolic rates of Bt resistant (Cry5dT and CryReg) third and fifth instar larvae and 1-7 day-old pupae to the susceptible (SeA) strain of same instar is probably due to the absence of Cry1C toxin. Berdegue *et al.* (1996) reported that Cry1C resistant *S. exigua* larva may degrade the toxin and repair the midgut by replacing damaged cells from regenerative nidi. Furthermore, if recovered larvae were not exposed to the toxin again, recovery would be permanent (Dulmage *et al.,* 1977; Chiang *et al.,* 1986; Berdegue *et al.,* 1996; Stapel *et al.,* 1998; Loeb e*t al.,* 2001). Also, Ramachandran *et al.* (1993) showed that the spruce budworm could recover repeatedly from effects of the Cry1A(a) toxin, reinfecting themselves if the toxin was still available or recovering to grow normally in its absence. Therefore since Cry5dT was exposed to toxin only on the first five days (neonate to first instar) it may have had a high metabolic rate as a result of detoxification. However, the body mass (0.0005 ± 0.001 g) and movement of neonates precluded testing this hypothesis.

Because reduced binding was not the major mechanism of resistance in *S. exigua* (Moar *et al.,* 1995), the high metabolic rate evident in CryonT is probably associated with some other resistance mechanism. For example, Loeb *et al.* (2001) examined

regeneration of cultured midgut cells in larvae of *Heliothis virescens* after exposure to sub lethal doses of two strains of Bt. As the titres of Bt toxins increased, more mature midgut cells were destroyed; simultaneously, the number of stem and differentiating cells increased rapidly. These results suggest that increased metabolic rate of CryonT may be related to the replacement of midgut cells in resistant larvae.

The metabolic rate of insect pupae such as *Drosophila, Tenebrio* and *Phormia* exhibit a characteristic "U-shaped" curve during the pupal stage. An increased metabolic rate during the last larval instar and early pupal stage is followed by a reduction in metabolic rate to a minimum at mid pupal stage and a steady increase in metabolic rate prior to adult emergence (Park *et al*., 1960). Similar U-shaped $\dot{V}_{O_2}$ curves were observed in this study (Fig. 2.4 and 2.5). CryonT pupae had significantly greater $\dot{V}_{O_2}$ on the first pupal day compared with the other treatment groups. Prior to pupation, the midgut contents of *S. exigua* are excreted. It is possible that all of the Cry1C toxin may not have been excreted and an active component of the toxin could have flowed into the haemocoel from the midgut affecting the central nervous system directly or indirectly. Energy may have been diverted to detoxification or processes that eliminate the toxin, resulting in greater metabolic rate of CryonT pupae on the first pupal day. During the pupal stage, internal cells, tissues and organs undergo histolysis and histogenesis (Wasserthal, 1996). Therefore, the low metabolic rate at days three and four for all treatment groups suggest a transition phase, while the steady increase after that, results from remodelling of internal structures.

Despite statistically significant differences in larval and pupal RQ, the values fall within the range of lipid metabolism (Bartholomew, 1977). This is not uncommon because larvae feed continuously to store fat and other nutrients used in metamorphosis (Wasserthal, 1996). Similar low RQ values have been observed in isopods (B. Joos, personal communication) and diapausing lycinid pupae (A. Appel, personal communication).

Cry1C toxin affects the metabolic rate of larval and pupal *S. exigua;* metabolic rates in CryonT third instar larvae and 1 day-old pupae were increased compared with Cry5dT and CryReg. Because the same strain of Cry1C resistant *S. exigua* was used for exposure to Cry1C toxin, it is assumed that the metabolic responses were influenced by physiological processes and the period of time *S. exigua* was exposed to Cry1C toxin [i.e., lower metabolic rate when Cry1C resistant *S. exigua* was exposed to toxin for 5 d (Cry5dT) and when not exposed to toxin at all (CryReg), and a significant increase in metabolic rate when exposed to toxin continuously (CryonT)]. Gilliland *et al.* (2002) reported that there were no differences in Bt susceptibility and amino peptidase N activity in brush border membrane vesicles between first and third instar susceptible *S. exigua*. Therefore, the present results have direct implications for management decisions with first instar larvae, the instar for which most control measures are targeted. Continuous exposure to toxin results in increased metabolic rate and decreased body mass which could reduce fitness and therefore limit the spread of resistance. Increased developmental time could also decrease fitness by exposing larvae to predators and parasites for a longer period thereby increasing the chance of mortality. No fitness costs were detected with CryReg compared with the genetically susceptible SeA strain and Moar *et al.* (1995)

reported that rearing Cry1C resistant *S. exigua* on untreated diet for several generations resulted in a similar resistance ratio to a population selected with toxin for 5 days (Cry5dT) for multiple generations. Therefore, the present results have implications for resistance management strategies such as refugia. Transgenic Bt crops may therefore offer a better alternative to refugia in some cases.

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Table 2.1. Treatment groups of resistant and susceptible strains of *S. exigua*

Genetic Strain	Toxin treatment*	Code
Susceptible	None	SeA
Cry1C Resistant	None	CryReg
Cry1C Resistant	First 5 d after egg hatched	Cry5dT
Cry1C Resistant	Continuously until pupation	CryonT

*Cry1C toxin incorporated into normal artificial diet (Chalfant, 1975) at 320 µg toxin per g diet

Table 2.2. ANOVA table for (A) $\dot{V}_{O_2}$ (ml $g^{-1}h^{-1}$), (B) $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$), (C) Mass (g) and (D) RQ of third and fifth instar larvae of Cry1C resistant and susceptible strains of *S. exigua*

	A) $\dot{V}_{O_2}$ (ml g^{-1} h^{-1})					B) $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)				
Source	df	SS	MS	F	P	df	SS	MS	F	P
Instar	1	0.5399	0.5399	8.27	0.0048	1	0.2492	0.2492	10.29	0.0017
Treatment group	3	0.6449	0.2150	3.29	0.0232	3	0.5506	0.1835	7.57	0.0001
Instar*Treatment group	3	0.7815	0.2605	3.99	0.0096	3	0.0889	0.0296	1.22	0.3050
Error	112	7.3080	0.0652			112	2.7138	0.0242		

	C) Mass					D) RQ				
Source	df	SS	MS	F	P	df	SS	MS	F	P
Instar	1	0.9380	0.9380	1992.6	0.0001	1	0.0108	0.0108	0.39	0.5320
Treatment group	3	0.0551	0.0184	39.0	0.0001	3	0.1143	0.0387	1.41	0.2426
Instar*Treatment group	3	0.0372	0.0189	26.4	0.0001	3	0.0488	0.0163	0.59	0.6200
Error	112	0.0527	0.0005			112	3.0671	0.0274		

Table 2.3. Sources of variation, *F*-statistics, degrees of freedom (numerator, denominator), and probabilities for A) $\dot{V}_{O_2}$ (ml $g^{-1}h^{-1}$), B) $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$), C) Mass (g) and D) RQ of 1-7 day-old pupae of Cry1C resistant and susceptible strains of *S.exigua*

	A) $\dot{V}_{O_2}$ (ml $g^{-1}h^{-1}$)			B) $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)		
Source	F	df	P	F	df	P
Day	40.41	6,667	0.0001	93.18	6,667	0.0001
Treatment group	2.11	3,667	0.0097	1.30	3,667	0.0273
Day*Treatment group	3.39	18,667	0.0001	1.35	18,667	0.1501

	C) Mass(g)			D) RQ		
Source	F	df	P	F	df	P
Day	8.93	6,667	0.0001	8.86	6,667	0.0001
Treatment group	512.64	3,667	0.0001	6.83	3,667	0.0002
Day*Treatment group	0.87	18,667	0.6123	1.41	18,667	0.1200

FIGURE LEGENDS

Fig. 2.1. $\dot{V}_{O_2}$ (ml $g^{-1}h^{-1}$) for third and fifth instar larvae.

* significantly different $P \leq 0.05$

Fig. 2.2. $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$) for third and fifth instar larvae.

* significantly different $P \leq 0.05$

Fig. 2.3. Mass of third and fifth instar larvae.

* significantly different $P \leq 0.05$ (third and fifth instar larvae)

Fig. 2.4. Variation in $\dot{V}_{O_2}$ (ml $g^{-1}h^{-1}$) consumed by pupae of Cry1C susceptible and resistant *S. exigua*.

Fig. 2.5. Variation in $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$) consumed by pupae of Cry1C susceptible and resistant *S. exigua*.

Fig. 2.6. Changes in mass of pupae of Cry1C susceptible and resistant *S. exigua*.

* significantly different $P \leq 0.05$

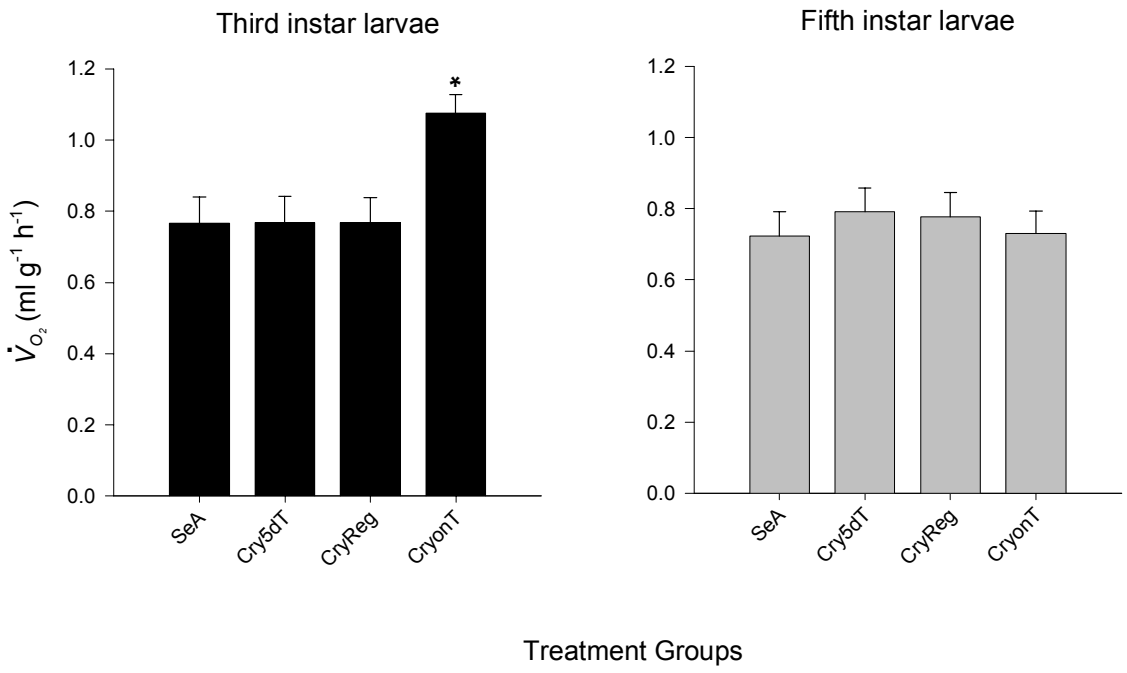
Third instar larvae
Fifth instar larvae
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
SeA
Cry5dT
CryReg
CryonT
Treatment Groups

Fig. 2.1

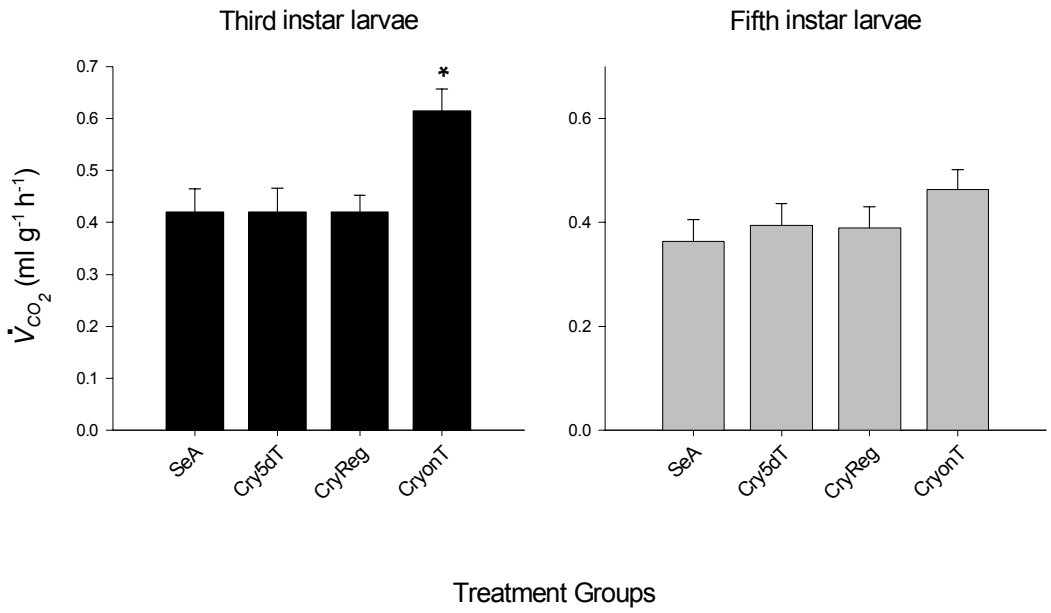
Third instar larvae
Fifth instar larvae
$\dot{V}_{CO_2}$ (ml g^{-1} h^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
*
SeA
Cry5dT
CryReg
CryonT
Treatment Groups

Fig. 2.2

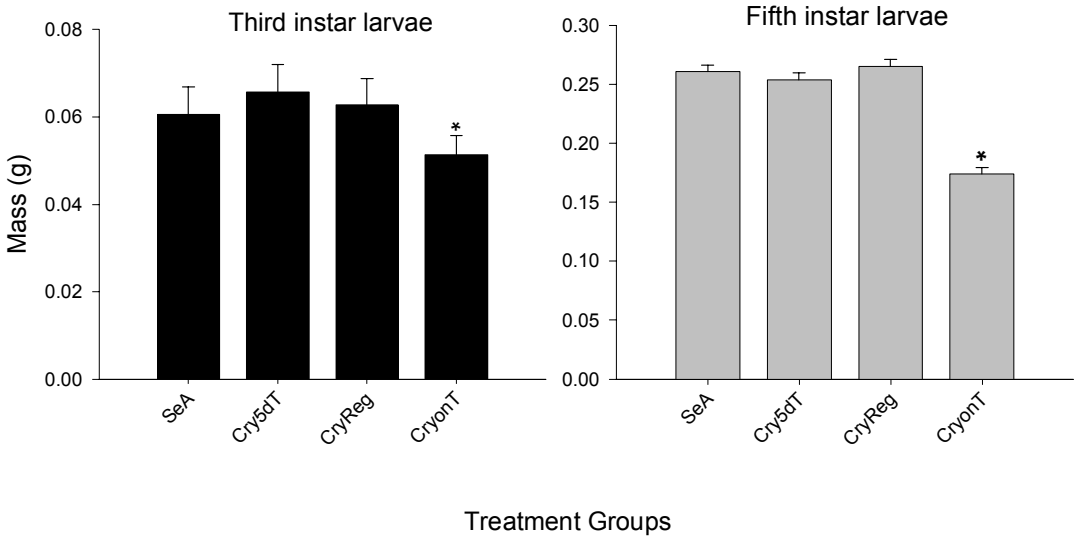
Third instar larvae
Fifth instar larvae
Mass (g)
0.08
0.06
0.04
0.02
0.00
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
SeA
Cry5dT
CryReg
CryonT
Treatment Groups

Fig. 2.3.

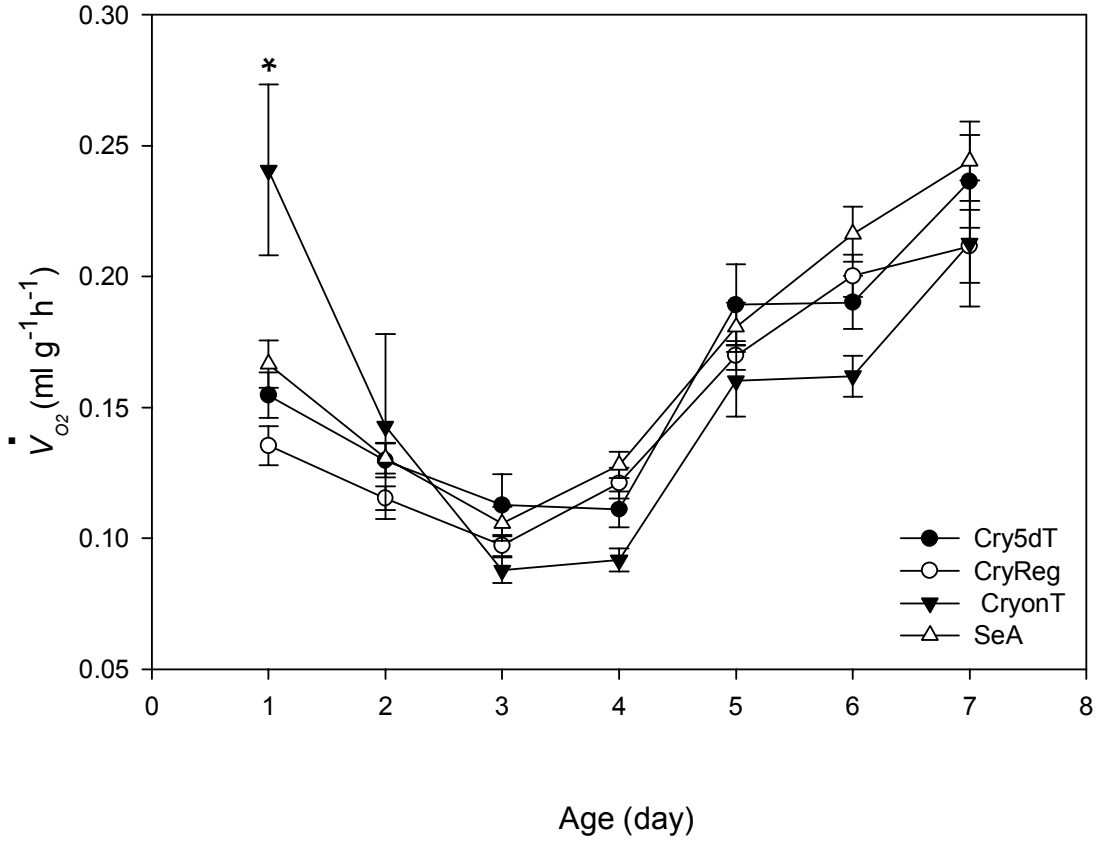
0.30
0.25
0.20
0.15
0.10
0.05
*
$\dot{V}_{O2}$ (ml g^{-1}h^{-1})
0
1
2
3
4
5
6
7
8
Age (day)
Cry5dT
CryReg
CryonT
SeA

Fig. 2.4.

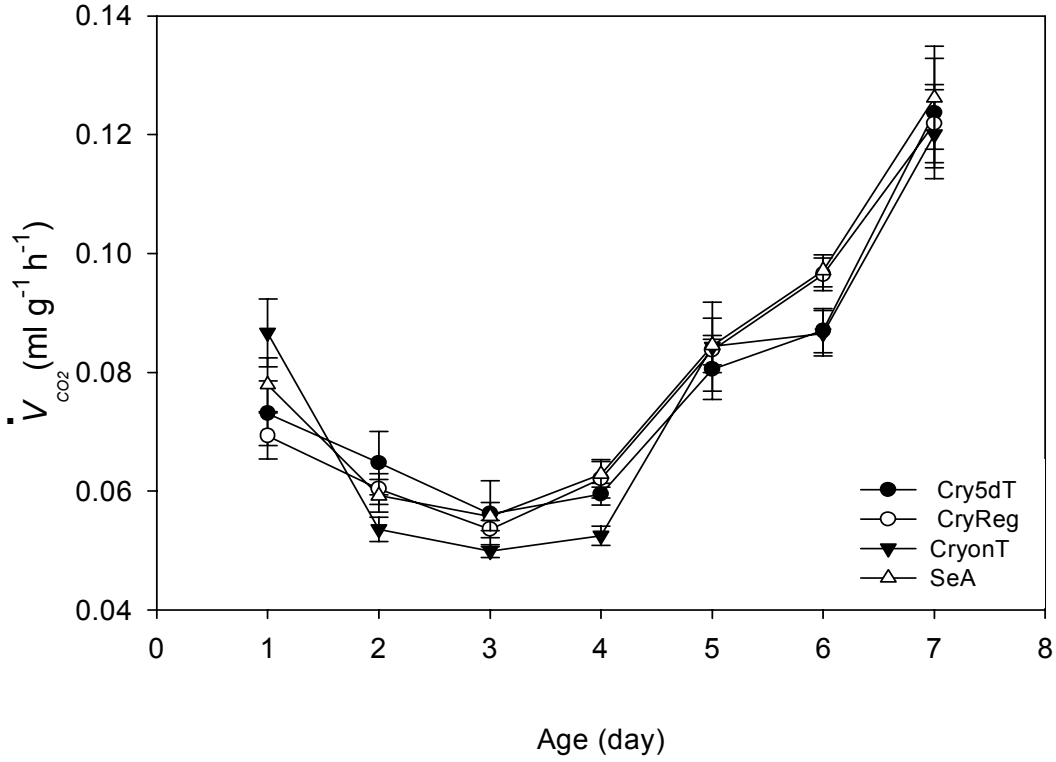
0.14
0.12
0.10
0.08
0.06
0.04
$\dot{V}_{CO2}$ (ml g^{-1} h^{-1})
0
1
2
3
4
5
6
7
8
Age (day)
Cry5dT
CryReg
CryonT
SeA

Fig. 2.5.

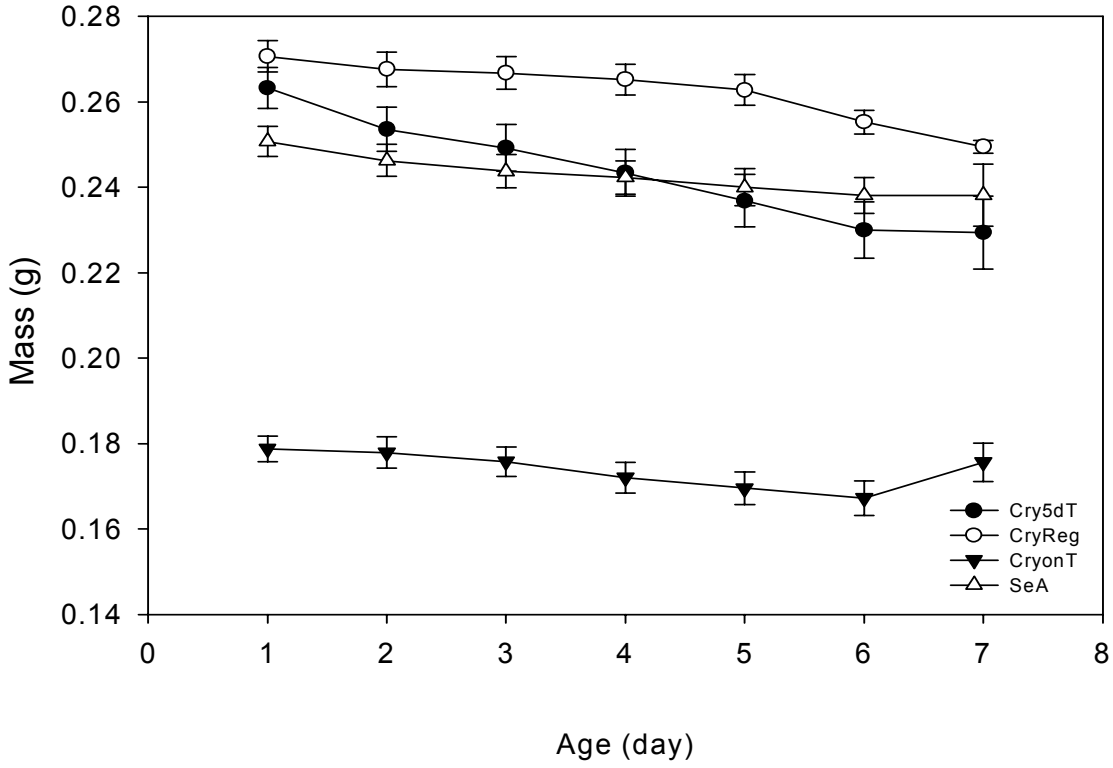
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
Mass (g)
0
1
2
3
4
5
6
7
8
Age (day)
Cry5dT
CryReg
CryonT
SeA

Fig. 2.6.

CHAPTER 3: DISCONTINUOUS GAS EXCHANGE PATTERNS OF BEET ARMYWORM PUPAE, *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE) EFFECTS OF *BACILLUS THURINGIENSIS* CRY1C TOXIN, PUPAL AGE, AND TEMPERATURE

INTRODUCTION

The discontinuous gas exchange cycle (DGC), first described in detail in lepidopteran pupae (Levy and Schneiderman, 1966), has been reported in a number of insects including ants (Lighton and Berrigan, 1995; Vogt and Appel, 2000), beetles (Lighton, 1988, 1991), cockroaches (Kestler, 1991; Dingha et al.,in press) and grasshoppers (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993). The DGC has also been detected in non-insect arthropod groups, including centipedes (Klok *et al*., 2002), mites (Lighton and Duncan, 1995), solphugids (Lighton and Fielden, 1996) and ticks (Lighton and Fielden, 1995). DGC's generally consist of three phases: a closed (C) phase during which the spiracular valves are tightly closed and gas exchange is minimal/negligible; followed by a flutter (F) phase where the spiracles open and close rapidly enabling gases, particularly O_2 to pass through by convection and diffusion; and finally an open (O) phase where the spiracles are completely opened and the bulk of CO_2 is released from the tracheal system (Schneiderman, 1960; Lighton, 1994; 1996). The closed, flutter and open phases together makeup a cycle. Considerable variation in the

duration of cycles have been observed (Wasserthal, 1996); for example, DGC durations range from less than 5 min in some ant (Lighton and Berrigan, 1995), to 15 min in tok-tok beetles, *Psammodes striatus* (Lighton, 1988), to > 40 minutes in the keratin beetle, *Omorgus radula* (Bosch *et al*., 2000).

Initially, it was assumed the DGC evolved primarily as a water conservation mechanism (Kestler, 1985; Hadley, 1994; Lighton, 1996) because respiratory water loss is minimized when the spiracles are closed or fluttering. This hypothesis has been criticized because insects abandon the DGC when it is most needed for water conservation. For example, Hardley and Quinlan, (1993) showed that the lubber grasshopper, *Romalea guttata*, abandoned the DGC when dehydrated. Chappell and Rogowitz (2000) argue that at high temperatures, with greater metabolic rates resulting in increase tracheal saturation pressure, the DGC should be most pronounced. Instead, they found that at high temperatures the eucalytus boring beetle, *Phorocantha* spp., did not exhibit a DGC. Also, Rourke (2000) found that the California grasshopper, *Melanoplus sanguinipes,* abandoned the DGC at high temperature. Alternatively, it has been suggested that the DGC may have evolved as a means to enhance the efficacy of gaseous exchange in hypoxic and/or hypercapnic environments (Lighton and Berrigan, 1995; Lighton, 1996). However, Chown and Holter (2000) observed that adult *Aphodius fossor*, a beetle that inhabits moist, anoxic and hypercapnic dung pats, progressively abandoned the DGC when exposed to declining oxygen concentrations. More recently, Hetz and Bradley (2005) proposed a third possible explanation suggesting that insects breathe discontinuously when at rest to avoid oxygen toxicity. Their hypothesis is based

on the fact that exposure of tissues to high levels of O_2 is unusual among biological systems and that high levels of O_2 can cause oxidative damage. Consequently, it is O_2 regulation that produces the observed discontinuous release of CO_2.

There is considerable variation in the characteristics of the discontinuous gas exchange cycles (Chown, 2001; Marais and Chown, 2003). Variation may be found in the CO_2 release pattern (Lighton and Berrigan, 1995), or duration of the phases (Lighton, 1988, 1990, 1998). It is likely that many of these variations could be responses to environmental stressors (e.g., extreme temperatures, exposure to toxins, hypoxia, hypercapnia, desiccation and starvation) and developmental requirements (e.g., oxygen and water). Furthermore, environmental stress and changes in development may alter metabolic rates (Djawdan *et al*., 1997) and could change the time interval between openings of spiracles (Greenberg and Ar, 1996), thereby, affecting the DGC pattern. For example, in the dog tick, *Dermacentor variabilis,* blood feeding altered CO_2 emission pattern of adult females from DGC (days 1-6) to continuous CO_2 release during the final stages (days 9-11) of engorgement (Fielden *et al*., 1999). Despite all that is known about the DGC of insects and other arthropods, the effects of stressors such as toxins and developmental period (age) on the DGC pattern in pupae has not been widely studied.

Pupae of the beet armyworm, *Spodotera exigua* (Hübner) (Lepidoptera: Noctuidae), overwinter buried within the soil without diapausing (Fey and Carranza, 1973). Larvae damage most vegetable crops (Gho *et al*., 1993); however, effective control has been obtained using *Bacillus thuringiensis* (Berliner) (Bt). Activated toxins bind to specific receptors on the midgut epithelial membrane forming pores that result in

osmotic lysis of the midgut epithelial cells and eventually insect death (Knowles and Ellar, 1987). Previous examination of metabolic rates in *S. exigua* pupae using closed system respirometry (Dingha *et al*., 2004) reported increased metabolic rates in a *B. thuringiensis* Cry1C resistant strain reared continuously on toxin compared with the same strain unexposed to Bt. toxin and significant differences in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ throughout the pupal developmental period in both strains. Based on these findings, I hypothesized that there would be variations in the duration and amount of CO_2 emitted during the closed, flutter and open phases over the pupal developmental period between pupae stressed by previous exposure to toxin and unexposed pupae. In addition, I hypothesized that *S. exigua* would show a pronounced DGC only at lower temperatures and continuous cycling of CO_2 release at higher temperatures. Also, increased temperature generally causes metabolic rate to rise, the frequency of spiracular opening would increase at higher temperatures as observed in the eucalyptus-boring beetle and a California grasshopper (Chappell and Rogowitz, 2000; Rourke, 2000). I also describe changes of CO_2 release patterns observed in *S. exigua* pupae in response to previous exposure to *B. thuringiensis* and at several temperatures.

MATERIALS AND METHODS

Insects, toxin and treatment

Two strains of *S. exigua* were used for this study: a Cry1C susceptible strain and a Cry1C resistant strain. Both colonies were established at Auburn University, Alabama, USA, with insects collected from cotton (Moar *et al*., 1995). The susceptible colony was maintained on artificial diet (Chalfant, 1975) while the resistant colony was reared on

artificial diet containing 320µg Cry1C toxin per g diet. The Cry1C toxin from Bt was expressed in a Cry (-) strain of Bt, purified, trypsin-activated and incorporated into artificial diet as previously described (Moar *et a*l., 1995). Insects were reared at 28 $\pm$ 1^{o}C with a LD 16:8h photocycle. Previous results indicated that the pupae of Cry1C resistant strain reared on artificial diet alone without prior exposure to toxin during larval development did not differ in metabolic rates from the Cry1C susceptible strain (Dingha *et al*., 2004). In addition, preliminary experiments indicated that there were no significant differences between unexposed Cry1C resistant and susceptible strains in metabolic rate and DGC characteristics over developmental time. Therefore, pupae of both strains whose larvae were unexposed to Cry1C toxin represent the unexposed (no toxin) treatment group. The Cry1C resistant strain reared continuously on 320µg Cry1C toxin per g diet was the toxin-exposed treatment group. The Cry1C susceptible strain had never been exposed to toxin and was susceptible to the toxin. Therefore, the susceptible strain larvae were not exposed to toxin because at even low doses most died and the few (<10%) that survived did not develop to the pupal stage.

Respirometry and metabolic rate measurement

A flow through respirometry system was used to measure CO_2 emission from 1-7 day-old pupae at 10, 15, 20 and 25^{o}C. Outside air was scrubbed of CO_2 and H_2O using a Whatman purge gas Generator (Whatman, Inc., Haverhill MA, USA), drawn through a computer-controlled base lining system, a Li–Cor CO_2 and H_2O analyzer (LI-6262; LiCor Inc., Lincoln, Nebraska, USA) and a Side-Track mass flow meter (Sierra Instruments Inc., Monterey, CA, USA) with a pump (Gast Mfg. Corp., Benton Harbor, MI, USA) at a

flow rate of 100 ml/min at STP. The gas sample passed through the system and data from the CO_2 analyzer was recorded using DATACAN V (Version 5.2; Sable Systems, Henderson, NV, USA) software. The analyzer was calibrated with 94.9 ppm span gas (Air Products, Inc.) weekly. Measurements were made on individual pupae that had been weighed previously on an electronic balance to the nearest 0.01mg and placed in a glass respirometer chamber. The respirometer was housed in a Sable Systems (Henderson, NV, USA) PT-1 Peltier-effect temperature-controlled cabinet at the various temperatures. Each recording lasted 3-4 h after which the pupae were weighed again. The procedure was performed daily at the same time of day on all pupae for the entire 7 day pupal stadium. Each treatment group initially consisted of 10 pupae, of these, a minimum of 4 pupae were each recorded daily for 7 days. Because some pupae did not cycle every day, and only 4 pupae could be recorded each day, we repeated the experiment four times with new groups of pupae over 5 generations. Usable recordings were obtained from 5-7 pupae for each of the 7 days. A minimum of 5 cycles were analyzed for each pupae on each day. The characteristics of the discontinuous gas exchange cycle (DGC) were calculated for each individual on each day as follows: the mean rate of CO_2 emission for each phase of each cycle ($\dot{V}_{CO_2}$ ml/h) is the volume of CO_2 emitted during that phase, obtained by calculating the area under the CO_2 emission curve divided by phase duration and then expressed as a mass-specific value resulting in units of (ml g^{-1} h^{-1}). Mass scaling of the cycles and its characteristics were determined using least squares linear regression. The phase duration is the time taken for CO_2 to be emitted during that phase. All calculations of rate and duration of CO_2 release were carried out using DATACAN V

software. Overall metabolic rate or $\dot{V}_{CO_2}$ ml g^{-1} h^{-1} for the entire 7 day period was also calculated. Respiratory and cuticular water loss was calculated from recordings at 10°C because DGC was most frequent at this temperature. Respiratory water loss was calculated as the difference between cuticular and total water loss. Water loss through the cuticle or CP was calculated as the microgram of water lost during the entire recording per unit body surface area (cm^2) per unit time (h) per unit saturation deficiet (mmHg). Surface area was estimated for each pupae using Meeh's formular (Meeh, 1897):

$S = 12\ M^{2/3}$,

Where *S*, body surface area (cm^2) and *M*, initial mass (g) (Edney and McFarlane, 1974).

Data analysis

Data were subjected to analysis of covariance (ANCOVA) to estimate the mass scaling coefficient between metabolic rate and body mass for each treatment group. Regression analysis was performed if there was a significant effect of mass to estimate the slope of the Log $\dot{V}_{CO_2}$ (mg/h) and Log mass (g) relationship. A modified *T*-test ($P \leq 0.05$) was used to test if the slope was significantly different than 1 (Sokal and Rohlf, 1981). The slope of this relationship is the mass scaling coefficient and it was used to appropriately correct for the effect of body mass on $\dot{V}_{CO_2}$ resulting in mass independent metabolic rate (MIMR). In the gas exchange characteristics, the sample size was represented by 5-7 pupae for each of the 7 days with a minimum of 5 cycles for each pupae on each day. Because each DGC represented a replicate of the recording,

comparison between strains over time was analyzed using repeated measures analysis of variance (ANOVA). The test of fixed effects (PROC Mixed Procedure, ANOVA; Khattree and Naik, 1999, SAS Institute, 1996) was used to determine the effect of day, treatment group and the day by treatment group interaction on $\dot{V}_{CO_2}$. We used the ANOVA general linear model procedure (Proc GLM, ANOVA, SAS Institute, 1996), to compare overall (entire 7 day) treatment group effects. Mean separation was carried out using the Ryan-Einot Gabriel-Welsch Multiple Range test (REGWQ) that controls both type I and type II error. Polynomial regression of the form $y = y_o + ax + bx^2$, was used to describe the relationship between $\dot{V}_{CO_2}$ and pupal age (day) for each treatment group (SigmaPlot 8.0; SPSS, 2002). The regression coefficients between the two treatment groups were compared. Rate of water loss during each recording was calculated each day and for the entire 7 day experiment as the difference between initial and final mass and time spent in the respirometer and expressed as percentage of initial mass loss per hour. In measuring respiratory and cuticular water loss, we examined 5 pupae of each treatment group. Five to 7 DGC cycles were analyzed for each individual pupae. We tested for differences in water loss between the two treatment groups, among days and the treatment group by day interaction using ANOVA. A *T*-test was used if treatment groups were significantly different. A significance level of $P \leq 0.05$ was used throughout; data are expressed as means ± SEM.

RESULTS

The relationship of Log metabolic rate to Log body mass was not significantly different between treatment groups. The data were combined, regressed and had a slope

of 0.66 ± 0.054 which was significantly less than 1. Therefore, $\dot{V}_{CO_2}$ (ml/h) values were divided by body mass $^{0.66}$ to obtain mass independent metabolic rate (MIMR) in units of ml g^{-1} h^{-1}. Carbon dioxide emission pattern varied greatly with temperature in both toxin-exposed and unexposed *S. exigua* pupae. At 10°C the pattern was discontinuous and respiration became increasingly continuous when ambient temperature was raised in both strains (Fig. 3.1). The probability that *S. exigua* would respire discontinuously varied with temperature. At 10°C, approximately 80% of the individuals exhibited a DGC, 40% at 15°C, and less than 5% at 20°C. The closed, flutter and open phases together makeup one complete DGC. During the 7 day experiment, the three phases (closed, flutter and open) of the DGC pattern were distinct in pupae of both treatment groups at 10°C (Fig. 3.2). However, there were significant differences in the duration of the phases among days and between treatment groups with a significant day by treatment group interaction throughout the 7 day period (Table 3.1). The duration of the closed, flutter and open phases for the toxin exposed strain ranged from 9.5 ± 2.6 min on day 3 to 24.9 ± 2.9 min on day 2, 13.6 ± 2.2 min on day 7 to 35.9 ± 1.3 min on day 2, and 15.0 ± 3.8 min on day 6 to 30.0 ± 2.6 min on day 5, respectively. For the unexposed strain, the duration of the closed, flutter and open phases ranged from 5.6 ± 0.6 min on day 6 to 13.2 ± 1.6 min on day 6, 14.2 ± 1.2 min on day 1 to 20.7 ± 2.6 min on day 2, and 16.0 ± 1.3 min on day 1 to 20.9 ± 1.8 min on day 3, respectively (Table 3.2). Figs. 3.2 and 3.3A illustrate the significantly longer duration of the closed, flutter and open phases of pupae exposed to toxin continuously compared with untreated pupae during a complete DGC. Pupae exposed to toxin as larvae had closed, flutter and open phase durations 14 ± 4.0, 19 ± 2.3 and 12 ± 2.8%, respectively, greater than untreated pupae. Toxin-exposed pupae had

mean cycle durations of 60 ± 2.5 min whereas the cycle duration of unexposed pupae was 42 ± 1.8 min. $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) of closed, flutter and open phases varied significantly throughout the 7 day experiment and between treatment groups with a significant treatment group by day interaction (Table 3.3). $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) of the closed, flutter and open phases for the toxin exposed strain ranged from 0.0008 ± 0.0003 on day 3 to 0.013 ± 0.009 on day 1, 0.0012 ± 0.0004 on day 3 to 0.0124 ± 0.003 on day 7, and 0.04 ± 0.001 on day 4 to 0.079 ± 0.004 on day 1, respectively (Table 3.4). For the unexposed strain, $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) of the closed, flutter and open phases ranged from 0.0004 ± 0.0001 on day 3 to 0.004 ± 0.001 on day 7, 0.0012 ± 0.0002 on day 3 to 0.009 ± 0.002 on day 7, and 0.034 ± 0.003 on day 3 to 0.079 ± 0.004 on day 7, respectively (Table 3.4). Also, $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) for the flutter phase day by treatment group interaction was not significant (Table 3.2B). Fig. 4 shows the change in $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) over the pupal stadium in toxin exposed and unexposed pupae: greater CO_2 production early in the stadium followed by a decline at mid-stadium (days 3-4) and an increase late in the stadium (> day 4). On the first 3 days of the stadium, the amount of CO_2 produced by pupae exposed to toxin was almost twice that of the unexposed pupae. Furthermore, the minimum $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) was on day 4 for the exposed pupae and day 3 for unexposed pupae. Both curves were compared using polynomial regression (Fig. 3.4). The model was highly significant (P<0.0001) for both curves and the r^2 indicated a good fit. Combining all 7 days closed, fluttered and opened $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) (Fig. 3.3B), pupae exposed to toxin produced significantly more CO_2. Pupae exposed to toxin on average

produced 12 ± 52, 10 ± 43, and 37 ± 15% more CO_2 than untreated pupae in the closed, flutter and open phases, respectively. There was a significant difference in overall water loss between the two strains (F= 6.99; df= 1, 6; P<0.01), but there was no significant difference in water loss among days (P= 0.079) or for the strain by day interaction (P= 0.077). During a DGC, total water lose (respiratory plus cuticular) was greater in pupae whose larvae were exposed to toxin compared with the unexposed pupae (Table 3.5). Also, the percentage respiratory water lose during a complete DGC (closed, flutter and open phases) was 4.5% ± 1.3 for the resistant strain and 2.1% ± 2.4 for the susceptible strain. However, in both strains, cuticular transpiration (>93%) far exceeds respiratory transpiration (Table 3.5). Cuticular permeability values using Meeh (1897) surface area model estimates were significantly different (P<0.0001) between both strains. The mean cuticular permeability was 26.01 ± 1.9 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the resistant strain and 9.64 ± 0.9 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the susceptible strain

DISCUSSION

Temperature affects the metabolic rate of ectothermic organisms and acts as a stressor in insects when it drops below or increases above the insect's optimum temperature range (Willmer *et al*., 2000, Acar *et al.*, 2001). An important finding of this study is that both toxin exposed and unexposed *S. exigua* pupae exhibited DGC at 10^{o}C. Below 10^{o}C there were no recordings of a flutter or open phase for >45minutes and the CO_2 emission pattern remained steady through out this time (Dingha, personal observation). The pupae were alive because when disturbed, there was body movement. However, as temperatures increased above 10^{o}C the pattern changed markedly from DGC to cyclic CO_2 release (Shelton and Appel, 2001) to continuous respiration (Fig. 3.1). Similar results in which DGC was observed at lower temperatures have been reported by Chappell and Rogowitz (2000) for the eucalyptus beetle. Generally, DGC is displayed when CO_2 accumulates over time in the tracheal system and is periodically released when it reaches a critical threshold (Lighton, 1996). On the other hand, when CO_2 accumulates rapidly, the spiracles open more frequently resulting in continuous respiration. Therefore, this spiracular behaviour could be attributed to the fact that at lower temperatures, metabolism is lower and CO_2 production is reduced thus the spiracles remain closed and are opened only when the concentration of CO_2 within the trachea has increased to a critical threshold. However, at higher temperatures, with increased metabolism, more CO_2 is released from tissues per unit time.

Based on the amount of CO_2 produced, pupae whose larvae were reared continuously on Cry1C toxin had greater metabolic rates than pupae whose larvae were

not exposed to toxin. Dingha *et al*. (2004), using closed system respirometry at 25^{o}C also reported that Cry1C resistant *S. exigua* pupae from larvae exposed continuously to Cry1C toxin had higher metabolic rates compared with unexposed pupae. Also, Kramarz and Kafel (2003) reported increased metabolic rates in *S. exigua* pupae whose larvae were fed zinc. They suggest that elevated metabolic rate could be due to the presence of a stressor (such as Bt toxin or zinc) in which case, more energy may have been required for detoxification or processes that eliminate the stressor. Furthermore, in larvae of *Bombyx mori*, Bt stimulated cellular respiration and toxin-induced uncoupling of oxidative phosphorylation (Faust *et al*., 1974). Loeb *et al.* (2001) examined regeneration of cultured midgut cells in larvae of tobacco budworm, *Heliothis virescens*, after exposure to sublethal doses of two strains of Bt (AA1-9 and HD-73). As the titers of Bt toxins increased, more mature midgut cells were destroyed; simultaneously, the number of stem and differentiating cells increased rapidly. This result suggests that increased metabolic rate in pupae exposed to toxin may be related to the repair of midgut cells during histogenesis. Higher metabolic rates of pupae exposed to toxin was accompanied by DGC; however, unexposed pupae that had lower metabolic rates also exhibited DGC. Moreover, DGC was exhibited by both treatment groups on all days irrespective of metabolic rate. One possible explanation may be that CO_2 produced in both strains at 10^{o}C on all days never increased rapidly in the tissues and haemolymph thereby, increasing the time necessary to reach the hypercapnic or acidic set point that initiates opening of the spiracles. Although both strains exhibited the DGC on all days, there were variations in the phase durations among the days (Table 3.2 and 3.4). Keister and Buck (1961) found that pupae of the fly, *Phormia regina*, consume less oxygen 2 days

after puparium formation compared with the other days. Even though O_2 consumption was not measured in this experiment, these differences could be associated with oxygen requirement on a particular day. Therefore, study of insect metabolism and evaluation of the adaptive significance of gas exchange patterns must take developmental time into consideration because of the temporal variations in oxygen requirements.

Generally, the metabolic rates of both strains of *S. exigua* pupae were greater early in the pupal stadium followed by a reduction at mid-stadium and a steady increase prior to adult emergence resulting in a characteristic "U-shaped" curve (Fig. 3.4). It should be noted, however, that in this study we measured metabolic rate as the release of CO_2 rather than as oxygen consumption. Similar findings (which measured both CO_2 production and O_2 consumption) have been reported for other insect pupae including *S. exigua* (Parke and Buck, 1960; Dingha *et al*., 2004). This "U-shape" curve of metabolic rate over time is probably the result of first histolysis of larval tissues and then histogenesis of adult cells, tissues and organs (Wasserthal, 1996). The low metabolic rate for both treatment groups observed on days 3 and 4 (Fig. 3.4) may suggest a transition phase, while the steady increase after that, could result from remodeling of internal structures.

Following its characterization in lepidopteran pupae, the DGC was generally assumed to have evolved as a mechanism to enhance water conservation. This hypothesis was based on the notion that little or no respiratory water loss occurs during the closed and flutter phases with the majority of respiratory water loss occurring during the open phase (Lighton, 1996). The duration of the closed, flutter and open phases were

significantly longer in pupae exposed to toxin. In addition, respiratory and cuticular water lose was significantly greater in pupae exposed to toxin than unexposed pupae (Table 3.5). In *S. exigua* pupae exposed to toxin, the presence of a long closed and flutter phases should have resulted in decrease water loss. Instead, water loss was higher in this strain; it is possible that this could have resulted from the long duration of the open phase. In other studies, long flutter periods >23 min appear to be important in reducing water loss in arid adapted arthropods such as the ant *Cataglyphis bicolor* (Lighton and Wehner, 1993) and a trogid beetle (Bosch *et al*., 2000) where the need to conserve water is great. The rates of overall water loss in resistant and susceptible *S. exigua* pupae, when expressed per unit surface area and corrected for saturation deficit, fall within the range of values (0-30 $\mu g\ cm^{-2}\ h^{-1}\ mmHg^{-1}$) exhibited by arthropods that inhabit xeric environments (Edney, 1977). Even if water loss is insignificant in adult insects (Hadley, 1994; Chown and Holter, 2000), it could be very important in this immobile life history stage as the options available for replacing water loss are very limited. Most adult insects are active and can replenish body water by feeding and drinking. Consequently, toxin exposed pupae with higher cuticular permeability could be more susceptible to desiccation which may indirectly contribute to reduced fitness. Difference in cuticular permeability between susceptible and resistant *S. exigua* pupae could signify dissimilarity in the proportion or composition of lipids on the epicuticle. This could result from energy been diverted to detoxification of toxin instead of processes for making lipids and other important components of the cuticle.

The relative contribution of cuticular and respiratory transpiration to total water loss in some insects that exhibit DGC has been reported. For example, in two species of

ants, *Camponotus vicinus* (Lighton and Garrigan, 1995) and *C. bicolar* (Lighton and Wehner, 1993) at 25°C and the grasshoppers, *R. guttata* (Hadley and Quinlan, 1993) and *Taeniopoda eques* (Quinlan and Hadley, 1993) between 15 and 30°C, more than 92% of water loss was through cuticular transpiration. Cuticular transpiration accounted for >92% of total water loss in both resistant and susceptible *S. exigua* pupae at 10°C. These data clearly support the dominance of transcuticular water loss at low temperatures. Water loss associated with gas exchange may increase with activity and/or at higher temperatures (Hadley and Quinlan, 1993).

If the DGC is an evolutionary response to water conservation, it should be most pronounced at higher temperatures and at higher metabolic rates. These predictions are not true for *S. exigua* pupae. The alternate hypothesis that the DGC evolved as a means to facilitate gas exchange in hypercapnic and hypoxic environments (Lighton, 1996) is consistent with the biology of *S. exigua* pupae because pupation occurs in the soil and the pupae develops in a relatively anoxic and hypercapnic chamber constructed from sand and soil particles. However, these experiments were not conducted under such conditions; rather pupae were exposed to normoxic air with no CO_2 or water vapour. Therefore, the exhibition of DGC in *S. exigua* pupae is a response to temperature (a stressor) and probably not because of its habitat. Nevertheless, I do not know if *S. exigua* pupae would DGC or not under anoxic and hypercapnic conditions. To answer this question, further experiments need to be done. However, much of the work on DGC indicates that the cycles are adaptive and have evolved in response to one or several specific environmental conditions or stress (e.g., hypoxia, desiccation) (Kestler, 1985; Lighton, 1996, 1998).

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Table 3.1. Sources of variation, F-statistics, degrees of freedom (numerator, denominator), and probabilities for A) Closed, B) Flutter and C) Open phase duration (min) of 1-7 d pupae of S. *exigua* unexposed and exposed to Cry1C toxin

A) Closed (min)

Source	F	df	P
Day	7.45	6,178	0.0001
Treatment group	54.35	1,178	0.0001
Day*Treatment group	2.84	6,178	0.0115

B) Flutter (min)

Source	F	df	P
Day	4.21	6,178	0.0006
Treatment group	22.49	1,178	0.0001
Day*Treatment group	4.35	6,178	0.0004

C) Open (min)

Source	F	df	P
Day	3.46	6,178	0.0029
Treatment group	31.28	1,178	0.0001
Day*Treatment group	1.92	6,178	0.0080

Table 3.2 Mean ± SE of (A) closed, (B) flutter and (C) open phase duration (min) for *S. exigua* unexposed and exposed to Cry1C toxin on each day for the 7 d experimental period. Means within rows followed by the same letter are not significantly different at $P \leq 0.05$, REGWQ test.

	(A) Closed (min)		(B) Flutter (min)		(C) Open (min)	
Age (day)	Toxin exposed	Unexposed	Toxin exposed	Unexposed	Toxin exposed	Unexposed
1	23.27±4.498a	8.12±0.908b	26.43±5.696a	14.16±1.216b	23.30±2.247a	16.03±1.258b
2	24.87±2.942a	13.21±1.615b	35.87±1.320a	20.68±2.556a	25.03±0.956a	15.72±1.001b
3	16.33±2.429a	11.12±1.853a	30.79±4.567a	14.59±1.693b	21.25±2.870a	20.94±1.843a
4	12.56±3.381a	7.31±0.905b	23.94±5.167a	14.86±1.217b	24.20±4.202a	16.56±0.725b
5	23.02±8.831a	7.65±1.219b	33.32±1.539a	18.93±2.361b	25.98±2.586a	14.53±1.329b
6	9.47±2.491a	5.64±0.458b	13.54±4.701a	17.85±1.466a	15.01±3.774a	13.60±0.896a
7	10.93±1.621a	6.63±0.909b	13.63±2.199a	17.21±2.090a	22.75±1.448a	17.21±1.296b

Table 3.3. Sources of variation, F-statistics, degrees of freedom (numerator, denominator), and probabilities for A) Closed, B) Flutter and C) Open phases $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$) of 1-7 d pupae of S.exigua unexposed and exposed to Cry1C toxin

	A) Closed $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)			B) Flutter $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)			C) Open $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)		
Source	F	df	P	F	df	P	F	df	P
Day	5.23	6,178	0.0001	10.08	6,178	0.0001	15.34	6,178	0.0001
Treatment group	8.26	1,178	0.0045	6.74	1,178	0.0102	2.81	1,178	0.0095
Day*Treatment group	3.09	6,178	0.0066	1.26	6,178	0.2778	2.70	6,178	0.0157

Table 3.4. Mean ± SE of (A) closed, (B) flutter and (C) open phase $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$) for *S. exigua* unexposed and exposed to Cry1C toxin on each day for the 7 d experimental period. Means within rows followed by the same letter are not significantly different at $P \leq 0.05$, REGWQ

	(A) Closed $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)		(B) Flutter $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)		(C) Open $\dot{V}_{CO_2}$ (ml $g^{-1}h^{-1}$)	
Age (day)	Toxin exposed	Unexposed	Toxin exposed	Unexposed	Toxin exposed	Unexposed
1	0.0134±0.0085a	0.0027±0.0007b	0.0060±0.0006a	0.0052±0.0012a	0.0758±0.0043a	0.0516±0.0040b
2	0.0060±0.0041a	0.0016±0.0008a	0.0051±0.0010a	0.0018±0.0004b	0.0553±0.0034a	0.0420±0.0015b
3	0.0008±0.0003a	0.0004±0.0001a	0.0012±0.0004a	0.0012±0.0002a	0.0459±0.0043a	0.0339±0.0028b
4	0.0010±0.0005a	0.0005±0.0001a	0.0052±0.0014a	0.0038±0.0008a	0.0399±0.0018a	0.0360±0.0023a
5	0.0022±0.0013a	0.0015±0.0003a	0.0037±0.0011a	0.0031±0.0006a	0.0446±0.0153a	0.0477±0.0026a
6	0.0032±0.0010a	0.0021±0.0006a	0.0119±0.0031a	0.0051±0.0009b	0.0525±0.0124a	0.0612±0.0027a
7	0.0041±0.0009a	0.0043±0.0012a	0.0124±0.0026a	0.0092±0.0017a	0.0719±0.0083a	0.0786±0.0043a

Table 3.5. Mean (± SE) water loss rates of the discontinuous gas exchange cycle of pupae of *S. exigua* unexposed and exposed to Cry1C toxin (n=5)

Treatment group	Cuticular water loss (mg/h)			Respiratory water loss (mg/h)			Total water loss (mg/h)		
	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max
Toxin exposed pupae	0.616±0.29a	0.164	1.04	0.047±0.15a	0.0003	0.661	0.664±0.36a	0.168	1.550
Unexposed pupae	0.376±0.14b	0.216	0.593	0.010±0.14b	0.0004	0.047	0.386±0.15b	0.230	0.631

Min = Minimum

Max = Maximum

[a] Means within colums followed by the same letter are not significantly different at $P \leq 0.05$

FIGURE LEGENDS

Fig. 3.1. Typical recording exhibited by S. exigua pupae unexposed and exposed to Cry1C toxin, at different temperatures

Fig. 3.2. Effect of *Bacillus thuringiensis* on the DGC pattern in *S. exigua* pupae at 10°C

Fig. 3.3. (a)Variation in duration (minute) and (b) $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) of the DGC phases in *S. exigua* pupae unexposed and exposed to Cy1C toxin at 10°C. Error bars represent standard errors of the means

*significantly different $P \leq 0.05$

Fig. 3.4. Changes in $\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) in *S. exigua* pupae unexposed and exposed to Cy1C toxin over developmental age at 10°C. Error bars represent standard errors of the means

* Significantly different $P \leq 0.05$

For the pupae exposed to toxin, the regression equation was:

$\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) = 0.131($\pm$0.004) - 0.042($\pm$0.002) day + 0.005($\pm$0.0003) day^2

(F=182, df=2, 6, P=0.0001, r^2=0.989).

For unexposed pupae, the regression equation was:

$\dot{V}_{CO_2}$ (ml g^{-1} h^{-1}) = 0.081($\pm$0.003) - 0.026($\pm$0.002) day + 0.004($\pm$0. 0002) day^2

(F=267, df=2, 6, P=0.0001, r^2=0.993). The regressions are illustrated as the broken lines in Fig. 3.4.

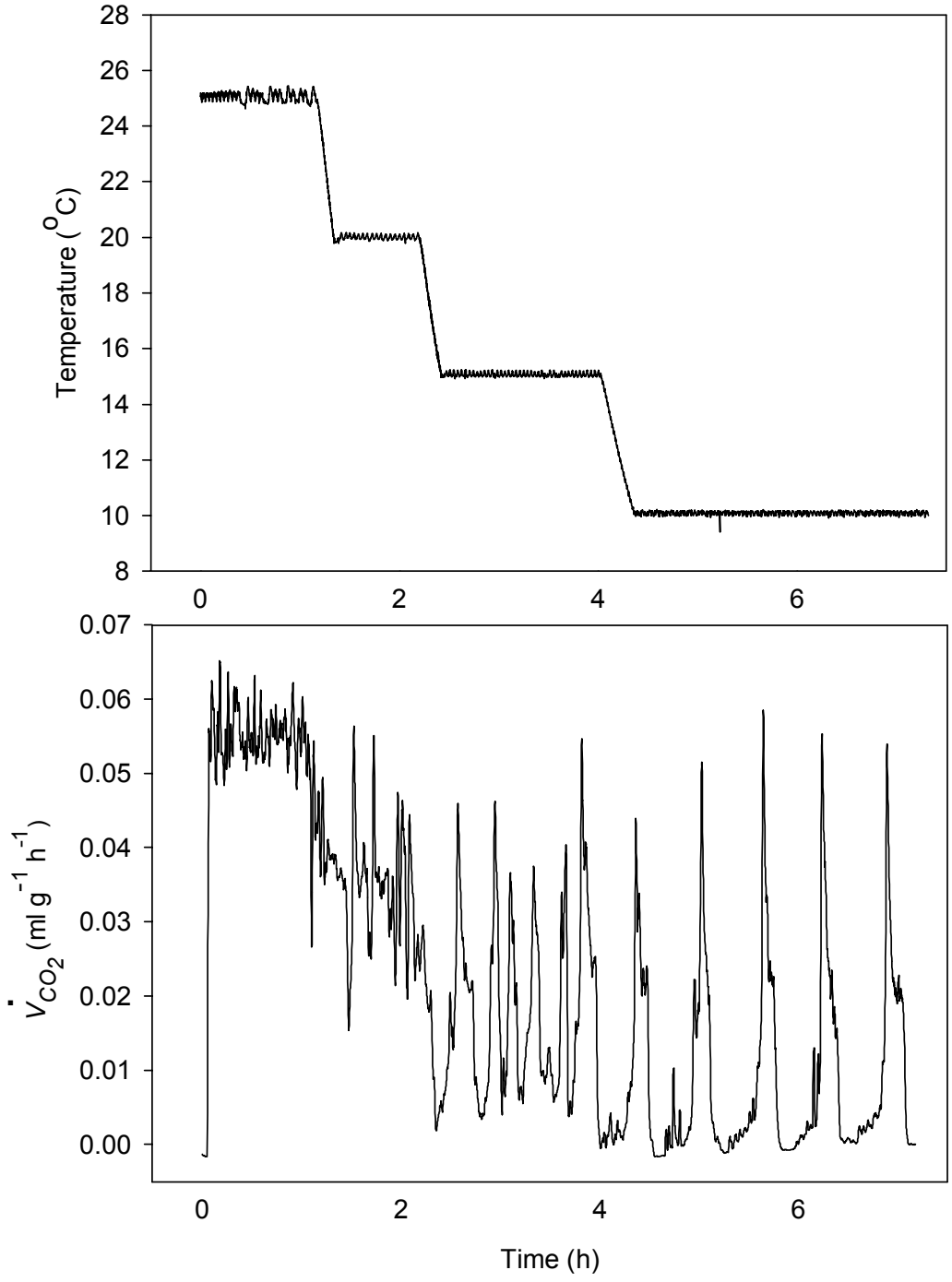

Temperature (°C)
28
26
24
22
20
18
16
14
12
10
8
0
2
4
6
$\dot{V}_{CO_2}$ (ml g^{-1} h^{-1})
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
2
4
6
Time (h)

Fig. 3.1

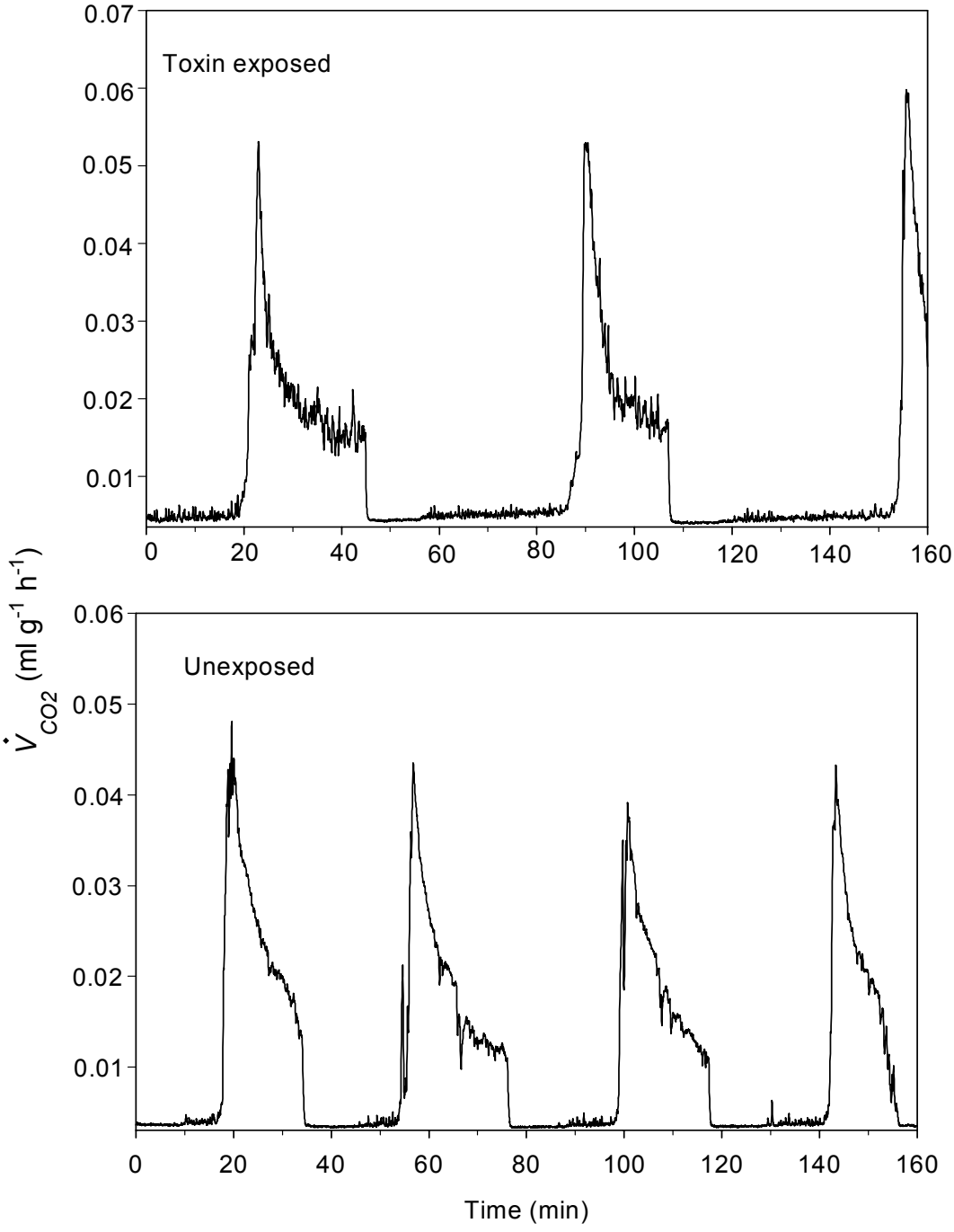
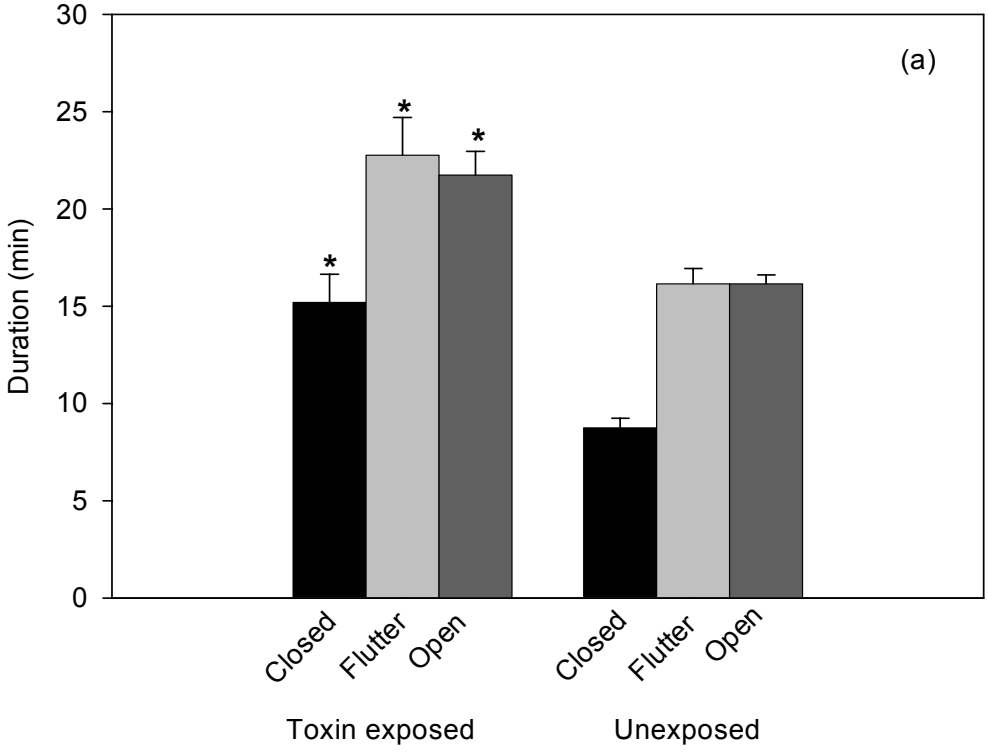

Toxin exposed
Unexposed
$\dot{V}_{CO2}$ (ml g^{-1} h^{-1})
Time (min)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
20
40
60
80
100
120
140
160

Fig. 3.2


(a)
Duration (min)
30
25
20
15
10
5
0
*
*
*
Closed
Flutter
Open
Closed
Flutter
Open
Toxin exposed
Unexposed

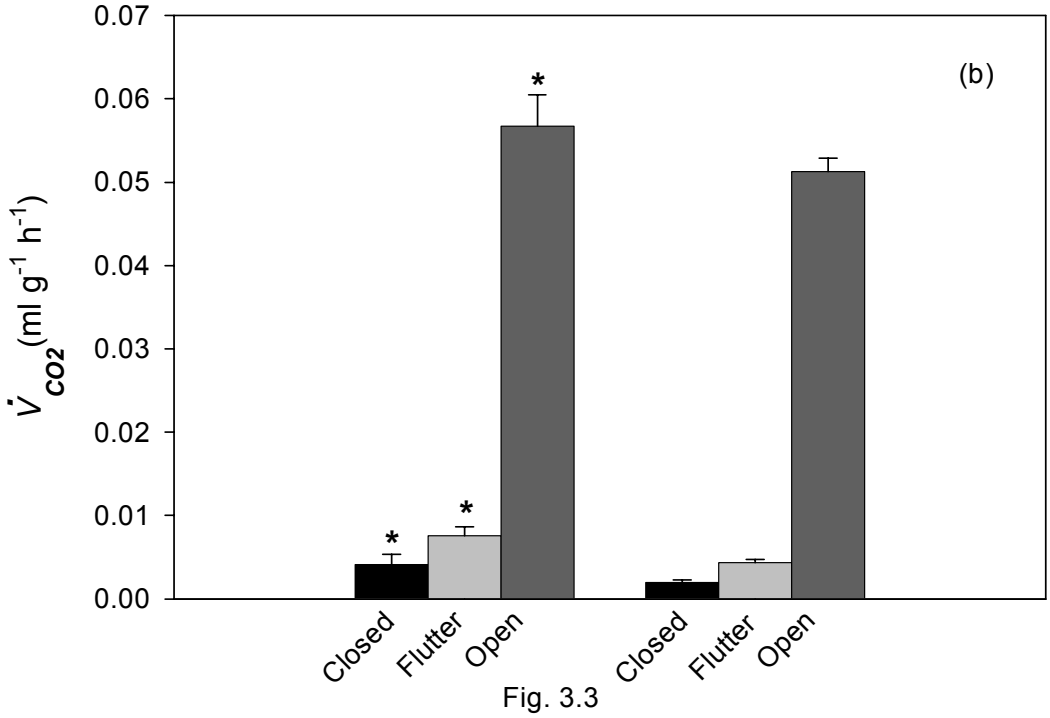

(b)
$\dot{V}_{CO2}$ (ml g^{-1} h^{-1})
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
*
Closed
Flutter
Open
Closed
Flutter
Open

Fig. 3.3

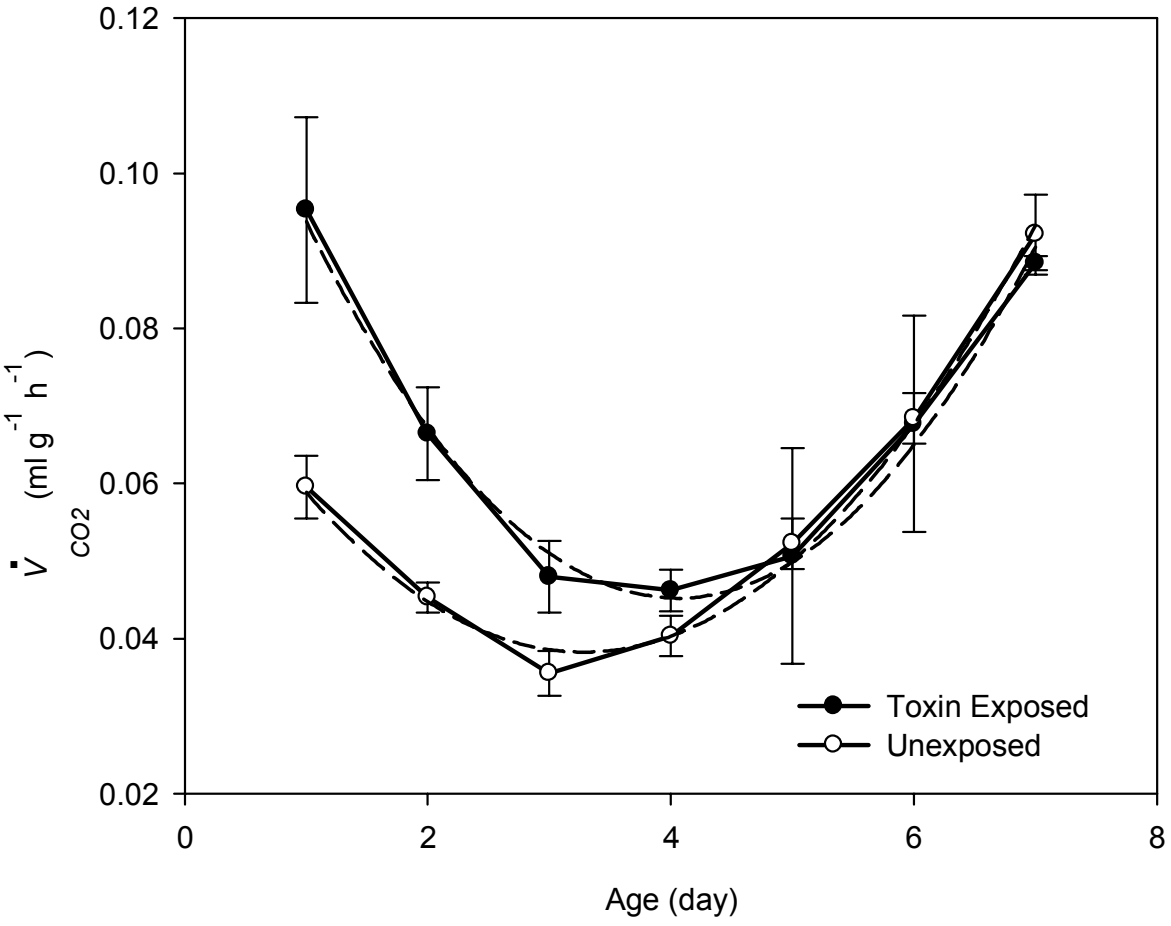

0.12
0.10
0.08
0.06
0.04
0.02
0
2
4
6
8
Age (day)
$\dot{V}_{CO2}$ (ml g^{-1} h^{-1})
Toxin Exposed
Unexposed

Fig. 3.4

CHAPTER 4: EFFECTS OF TEMPERATURE ON THE METABOLIC RATES OF INSECTICIDE RESISTANCE AND SUSCEPTIBLE GERMAN COCKROACH *BLATTELLA GERMANICA* (L.) (DICTYOPTERA: BLATTELIDAE)

INRODUCTION

Temperature is the most important determinant of metabolic rate in ectothermic animals (Cossins and Bowler, 1987; Angilletta et al., 2000; Gillooly et al., 2001) and controls nearly all physiological and biochemical processes (Huey and Berrigan, 2001). Increases in temperature generally result in increases in physiological processes including metabolic rate. It is widely acknowledged that metabolic rate in arthropods also varies as a consequence of locomotion (Rogowitz and Chappell, 2000), gender (Rogowitz and Chappell, 2000), altitude (Rourke, 2000), parasitism (Kolluru et al., 2002), water scarcity (Davis et al., 1999), climate (Nielsen et al., 1999), body mass (Gillooly et al., 2001), reproduction (Prestwich and Walker, 1981), and in the presence of insecticides and heavy metals (Kramarz and Kafel, 2003; Dingha et al., 2004).

Standard metabolic rate is a measure of the energetic cost of living, which in turn exerts a major influence on the fitness of organisms. Knowledge of the metabolic rate of an organism would provide an insight into the energetic cost thereby revealing the pattern of energy usage. To determine if there is an energetic cost associated with resistance to

insecticide, I measured the rate of O_2 consumption and CO_2 production in the German cockroach, *Blattella germanica* (L).

B. germanica is a world wide household pest, which may harbor and transmit human disease-causing pathogens. Their body parts and feces are also potent allergens to sensitive people. Pyrethroid insecticides are widely used for their control because of their effectiveness and low mammalian toxicity. However, control failures in some field populations have been reported as a result of the development of resistance (Cochran, 1989; Valles et al., 2000). It has been reported that resistance levels generally decline in the absence of insecticide selection (Tabashnik et al., 1994; Rahardja and Whalon, 1995), and that a decrease in resistance could be associated with increased fitness (Tabashnik et al., 1994). Therefore, I hypothesized that genetically resistant *B. germanica* not exposed to insecticide for several generations would have similar metabolic rates and respond to temperature change as a susceptible strain. I also described the effect of temperature on metabolic processes using Q_{10}, which is defined as the change in the rate of metabolism over $10^{o}C$ change in temperature. In addition, I obtained respiratory quotients (RQ), mass scaling relationships and compared these with those of other cockroaches.

METERIALS AND METHODS

Three strains of the German cockroach were used in this study. A black body mutant (Ross and Cochran, 1975) (black), and ACY (American Cyanamid Clifton, NY) were susceptible strains. Both strains have been reared in the laboratory without exposure to insecticide for over 40 generations. The insecticide resistant strain (Apyr-R) was collected from infested apartments in Opelika, Lee County, AL, U.S.A in 1999 after control failures with pyrethroid insecticides. This strain was subsequently selected with

permethrin for several generations (Wei et al., 2001; Pridgeon et al., 2002) but not during this experiment. All cockroaches were reared at 25 ± 2°C and 50 ± 10% RH with a photoperiod of 12L:12D. Dry dog chow and water were supplied *ad lib*.

One week old adult males from each strain were selected randomly and weighed on an electronic balance to the nearest 0.01 mg. Care was taken to avoid unnecessary stress to the cockroaches by allowing them to craw individually into weighing vials and respirometers constructed from 3 ml syringes (Becton, Dickinson and Company, Ruthford, NJ, U.S.A). Respirometers containing insects were connected to a manifold that provided dry, CO_2 free air at the rate of 100ml/min for ~ 10 min (Vogt and Appel, 1999). After flushing with dry CO_2-free air, the plunger was brought to the 2 ml gradation and the stopcock was adjusted to seal the respirometer. Respirometers were incubated in the presence of a video camera at 5, 10, 15, 20, 25, 30, 35, and 40 $\pm$ 2°C for 30 min. Temperature was checked with a calibrated mercury thermometer. Incubation took place under light from a 4 W red light bulb positioned ~50 cm above the experimental animals. The low level and color of the light facilitated filming of cockroaches during incubation using Panasonic GR-AX70u Compact VHS camera mounted on a tripod directly above the syringes containing cockroaches.

Oxygen depletion and carbon dioxide enrichment in each respirometer was determined using a Sable Systems TR-3 respirometry system (Sable Systems, Henderson, NV, USA). Outside air was scrubbed of CO_2 and H_2O using a Whatman purge gas Generator (Whatman, Inc., Haverhill MA, USA), drawn through a computer-controlled base lining system, a Li–Cor CO_2 and H_2O analyzer, a Sable Systems FC-1 Oxygen Analyzer, (LI-6262; LiCor Inc., Lincoln, Nebraska, USA) and a Side-Track mass flow

meter (Sierra Instruments Inc., Monterey, CA, USA) with a pump (GastMfg. Corp., Benton Harbor, MI, USA) at a rate of 100 ml/min at STP. The CO_2 analyzer was calibrated with 94.9 ppm span gas (Air Products, Inc.). The oxygen analyzer was internally zeroed and spanned to 20.94% O_2. From the 2 ml of air in a respirometer, 0.5 ml was injected into a glass T- injector port with a replaceable rubber septum and crimp seal installed anterior to the CO_2-H_2O analyzer. The gas sample passed through the system and data from the O_2 and CO_2 analyzers were recorded using DATACAN V (Version 5.2; Sable Systems, Henderson, NV, USA) software. At each temperature, we tested 5 male German cockroach of each treatment. This procedure was repeated three times at each of the different temperatures with all strains. Depletion (O_2) and enrichment (CO_2) volumes were integrated with respect to time. Rate of O_2 consumption was calculated as the volume (ml) of O_2 divided by insect body mass (g), and incubation time (min) resulting in $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}). Respiratory quotient (RQ) was calculated as the ratio of CO_2 produced ($\dot{V}_{CO_2}$) to O_2 consumed ($\dot{V}_{O_2}$).

Data analysis

Data were analyzed using Proc Mixed in SAS to determine the effects of cockroach strain on mean body mass, and to determine effects of temperature, strain, and the interaction of temperature and strain on $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}) and RQ. To elucidate possible interactions between effects of temperature, cockroach strain, and mass on $\dot{V}_{O_2}$ (ml h^{-1}), an additional analysis was conducted using all possible combinations of main effects and interactions. The random effects of block and the block by strain interaction were included in all analyses. Models were reduced based upon examination of *F*-statistics for

the main effects and interactions. Mean separation was carried out using LS means. Linear regressions were performed were appropriate.

RESULTS

Mean body mass of adult male cockroaches differed significantly among strains ($F = 30.0$, df = 2, 8, $P = 0.0002$); 0.0508 ± 0.0006, 0.0480 ± 0.0004, and 0.0543 ± 0.0005g for black, resistant, and ACY strains, respectively. The overall effect of strain on $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}) was not significant ($P > 0.05$), however, different strains reacted differently to increasing temperature ($F = 3.4$, df = 16, 161, $P < 0.0001$). Temperature effects were examined by regressing $\log_{10}$-transformed mass-specific $\dot{V}_{O_2}$ on temperature for each strain. For the black strain, the relationship yielded the equation:

$\log_{10}\dot{V}_{O_2}$ (ml g^{-1} h^{-1})=0.003(±0.036)-0.009(±0.001)Temperature

(F=42.1, df=1, 6, P=0.0006, r^2=0.875).

For the resistant strain, the equation was:

$\log_{10}\dot{V}_{O_2}$ (ml g^{-1} h^{-1})=0.044(±0.016)+0.0078(±0.0002)Temperature

(F=151.2, df =1, 6, P<0.0001, r^2=0.9618).

For the ACY strain, the relationship was:

$\log_{10}\dot{V}_{O_2}$ (ml g^{-1} h^{-1})=0.079 (±0.008)+0.0059(±0.0003)Temperature

(F=354.3, df=1, 6, P<0.0001, r^2=0.9833).

The effect of temperature on oxygen consumption of the three strains (black, resistant, and ACY) of *B. germanica* is presented graphically in (Fig. 4.1 A, B, and C respectively). Q_{10} was calculated by multiplying the slope of the first order log-linear regression of $\dot{V}_{O_2}$ (ml g^{-1} h^{-1}) on temperature by 10 and then taking the antilogarithm. This yields a

mean Q_{10} through out the experimental temperature range of 1.77, 1.19, and 1.15 for the black, resistant and ACY strains respectively. We fit a cubic equation to the temperature means (shown as solid lines in Fig. 4.1) to quantify changes in Q_{10} by differentiation of the polynomial equation (Lighton, 1989). The equation for the resistant strain was:

$\log_{10}\dot{V}_{O_2}$ ($mlg^{-1}h^{-1}$)=-0.007(±0.210)+0.053(±0.038)Temperature–0.002(±0.002)

$Temperature^2$+0.00004(±0.00003)$Temperature^3$

(F=47.4, df=3, 4, P=0.001, r^2=0.986)

For the black strain the relationship was:

$\log_{10}\dot{V}_{O_2}$ ($mlg^{-1}h^{-1}$)=0.542(±0.283)-0.061(±0.051)Temperature+0.003(±0.003)

$Temperature^2$-0.00002(±0.00005)$Temperature^3$

(F=44.8, df=3, 4, P=0.001, r^2=0.971)

For the ACY strain the equation was:

$\log_{10}\dot{V}_{O_2}$ ($mlg^{-1}h^{-1}$)=0.275(±0.092)+0.0023(±0.017)Temperature+0.001(±0.0008)

$Temperature^2$-0.00001(±0.00001)$Temperature^3$

(F=118.7, df=3, 4, P=0.002, r^2=0.988)

Strain alone did not explain a significant proportion of the variability in RQ (P>0.05) but the relationship between RQ and temperature differed among strains (F=2.8, df=16, 53, P=0.0025). Estimated least square mean RQ for each strain were regressed over temperature. Respiratory quotient generally increased with temperature up to about 25°C, declining thereafter in all the strains (Fig. 4.2A, B and C). RQ at 25°C and 15°C for the ACY and resistant strain were highly variable between blocks and were considered outlier for this analysis. The equation relating RQ to temperature in the black strain:

RQ=0.674(±0.090)+0.018(±0.009)Temperature–0.0005(±0.0002)Temperature^2

(*F*=8.6, *df*=2, 5, *P*=0.0239 r^2=0.629) (Fig. 4.2A).

For the resistant strain:

RQ=0.689(±0.084)+0.008(±0.009)Temperature–0.0003(±0.0002)Temperature^2

(*F*=4.23, *df* =2, 5, *P*=0.084 r^2=0.629) (Fig. 4.2B).

For the ACY strain:

RQ=0.562(±0.038)+0.027(±0.004)Temperature–0.0006(±0.0001)Temperature^2

(*F*=22.5, *df*=2, 4, *P*=0.0067 r^2=0.0067) (Fig. 4.2C).

All possible effects and interactions were included in a final model to determine their effect on raw $\dot{V}_{O_2}$ (ml h^{-1}). The reduced model included the effects of temperature (*F*=73.1, *df*=7, 357, *P*<0.0001), mass (*F*=12.1, *df*=1, 364, *P*=0.0006) and temperature by strain (*F*=2.97, df=16, 185, *P*=0.0002). Since mass did not interact with the other main effects in the model, reduced models using mass-corrected $\dot{V}_{O_2}$ are sufficient for predicting effects of temperature and strain on oxygen consumption. We used data at 25^{o}C to examine the effect of mass on the rate of oxygen consumption. The resulting relationship between $\dot{V}_{O_2}$ (ml h^{-1}) and mass for the resistant strain is:

$\log_{10}\dot{V}_{O_2}$ =-0.004(±0.013)+0.776(±0.256)$\log_{10}$Mass

(r^2=0.139), (Fig. 4.3A). For the black strain:

$\log_{10}\dot{V}_{O_2}$ =-0.0123(±0.021)+0.811(±0.407)$\log_{10}$Mass

(r^2=0.166), (Fig. 4.3B), and for the ACY strain:

$\log_{10}\dot{V}_{O_2}$ =-0.0148(±0.123)+8995(±0.224)$\log_{10}$Mass

(r^2=0.184) (Fig. 4.3C).

DISCUSSION

Insecticide resistance has been reported to decline in the absence of selection in some insects including, *Bacillus thuringiensis* resistant diamondback moth, *Plutella xylostella* (Tabashnik et al., 1994), and Colorado potato beetle, *Leptinotarsa decemlineata* (Rahardja and Whalon, 1995). Also in pyrethroid resistant *B. germanica*, resistance level declined from 140 to 1.6 fold in the absence of selection pressure after 15 generations (Cochran, 1993). This reversion may be associated with increased fitness (Tabashnik, 1994). For example, resistant beet armyworm pupae, *Spodoptera exigua* whose larvae were not exposed to Cry1C *B. thuringiensis* had similar metabolic rate as the susceptible strain whereas, pupae whose larvae were exposed continuously to toxin had greater metabolic rate (Dingha et al., 2004). Similarly, in this study, the metabolic rate of pyrethroid resistant *B. germanica* not exposed to insecticide for several generations was not significantly different from the susceptible stain. Hostetler et al. (1994) using closed system respirometry at 26^{o}C, and Dingha et al. (submitted) using flow through respirometry at 10^{o}C reported no significant difference between pyrethroid resistant and susceptible *B. germanica*. An explanation for the similarity in metabolic rate could be that the resistant strain of *B. germanic*a may have detoxification mechanisms that would increase metabolic rates, but require the presence of insecticide to induce production of the detoxifying enzymes (Terriere, 1983).

Even though metabolic rate was not significantly different among the strains, the three strains reacted differently to increasing temperatures. Generally, metabolic rate increase with increase temperature however, exceptions from the exponential model have been reported in fish (Jobling, 1994), and insects (Keister and John, 1973). In this study,

the black strain is an exception; at 5, 10 and 15°C, temperature had almost no effect on metabolism and the curve flattened (Fig. 4.1A). This may be due to reduced metabolic activities within this temperature range. At high temperatures of ~35°C, the respiration rate in all three strains appeared to decrease (Fig. 4.1.), indicating that 35°C may be a physiologically stressful temperature.

The supercooling point (SCP) of an organism refers to the temperature at which ice formation occurs spontaneously within that organism. Several factors including the presence of food in midgut, the existence of ice nucleating agents in the hemolymph and having contact with surface moisture could affect the supercooling points of insects (Somme, 1982). Results from preliminary experiments showed that the SCP of the resistant strain was significantly difference from the susceptible strain. The SCP was -9.71±0.47, -11.36±0.69 and -11.67±0.46 for the resistant, ACY and black strains respectively. The mechanisms of resistance to pyrethroid in *B. germanica* include P450 monooxygenase, hydrolase and altered sodium channels (Pridgeon et al., 2002). Wei et al. (2001) reported high levels of resistance in *B. germanica* to permethrin and deltamethrin, with resistance ratios of 97 and 480, respectively, compared with the susceptible strain. Previous exposure of the resistant strain to insecticide could have caused this difference.

Rate of oxygen consumption increased with body mass in *B. germanica* (Fig. 4.3A, B and C). The allometric exponent for the relationship between oxygen consumption and body mass was 0.78 for the resistant strain, 0.81 for the black strain and 0.89 for the ACY strain. $\dot{V}_{O_2}$ in insects has previously been shown to scale with exponents between 0.6 and 1.0 (Mispagel, 1981; Vogt and Appel, 2000). The relationship

between metabolic rate and body mass has been reported in some cockroaches for example, *Blaberus discoidalis*, where the exponent was 0.83 (Birchard and Arendse, 2001). Among several cockroach species, including *Periplaneta americana*, *Blatta orientalis*, *Leucophaea maderae* the exponent was 0.776 (Coelho and Moore, 1989). Gunn (1935) reported an exponent of 0.7-0.8 for *P. americana*, *B. orientalis*, and *B. germanica*. Therefore, our findings are similar to those of other cockroaches.

The relationship between metabolism and ambient temperature is best described by an exponential function (Q_{10}). Q_{10} depicts the magnitude of change in metabolic rate for a 10^{o}C change in temperature. Schmidt-Nielsen (1995), provides a great deal of information on the Q_{10} of the metabolic rate in insects, with values generally ranging from 1.5-3 (Prestwich and Walker, 1981; Davis et al., 1999; Rourke, 2000; Rogowitz and Chappell, 2000). The Q_{10} over the full temperature range (5-40^{o}C) for the three strains of *B. germanica* was 1.15 for the ACY, 1.19 for the resistant and 1.77 for the black. At lower temperatures (5-15^{o}C) the Q_{10} was 1.00, 1.14, and 1.18 for the black, ACY and resistant strains respectively. However, at higher temperature (20-35^{o}C), the Q_{10} was 1.20, 1.13, and 1.38 for the resistant, ACY and black strains respectively. Q_{10} is predicted to be higher at lower temperatures (Schmidt-Nielsen, 1995). In insects, this pattern is variable for example the Q_{10} for a grasshopper did not change with temperature (Harrison and Fewell, 1995). Similarly, Q_{10} did not vary with temperature in the resistant and ACY strains whereas in the black strain Q_{10} increased with temperature. Similar findings were reported for two species of desert cockroach, *Arenivaga apacha* and *A. investigata* (Cohen and Cohen, 1981). The black strain may be more adapted to hotter and drier environment and therefore has a higher temperature tolerance than the other two strains.

Although RQ is not a proof of the identity of a particular substrate used in respiration, it nevertheless allows assumption about the substrate metabolized (Withers, 1992). Generally, RQ values range from 1.00 for carbohydrate, 0.80 for protein, and 0.71 for lipid metabolism (Bartholomew, 1977). In *B. germanica*, RQs were affected by temperature. However, the RQ values suggest that lipid and protein served as the metabolic substrates.

In conclusion, information on the effect of temperature on metabolic rate of pyrethroid resistant and susceptible *B. germanica* indicates that different strains respond differently to temperature and this may directly or indirectly affect fitness.

FIGURE LEGEND

Fig. 4.1. Rate of oxygen consumption in pyrethroid resistant and susceptible strains of *B. germanica* at several temperatures. Broken line represents the first-order regression of log transformed oxygen consumption (ml g^{-1} h^{-1}) on temperature, solid lines represents the third-order regression. See text for equations.

Fig 4.2. Mass scaling of rate of oxygen consumption (ml h^{-1}) adjusted to 25^{o}C) of phyrethroid resistant and susceptible strains of *B. germanica*.

Fig 4.3. Influence of body mass on respiratory quotient (RQ) in phyrethroid resistant and susceptible strains of *B. germanica*.

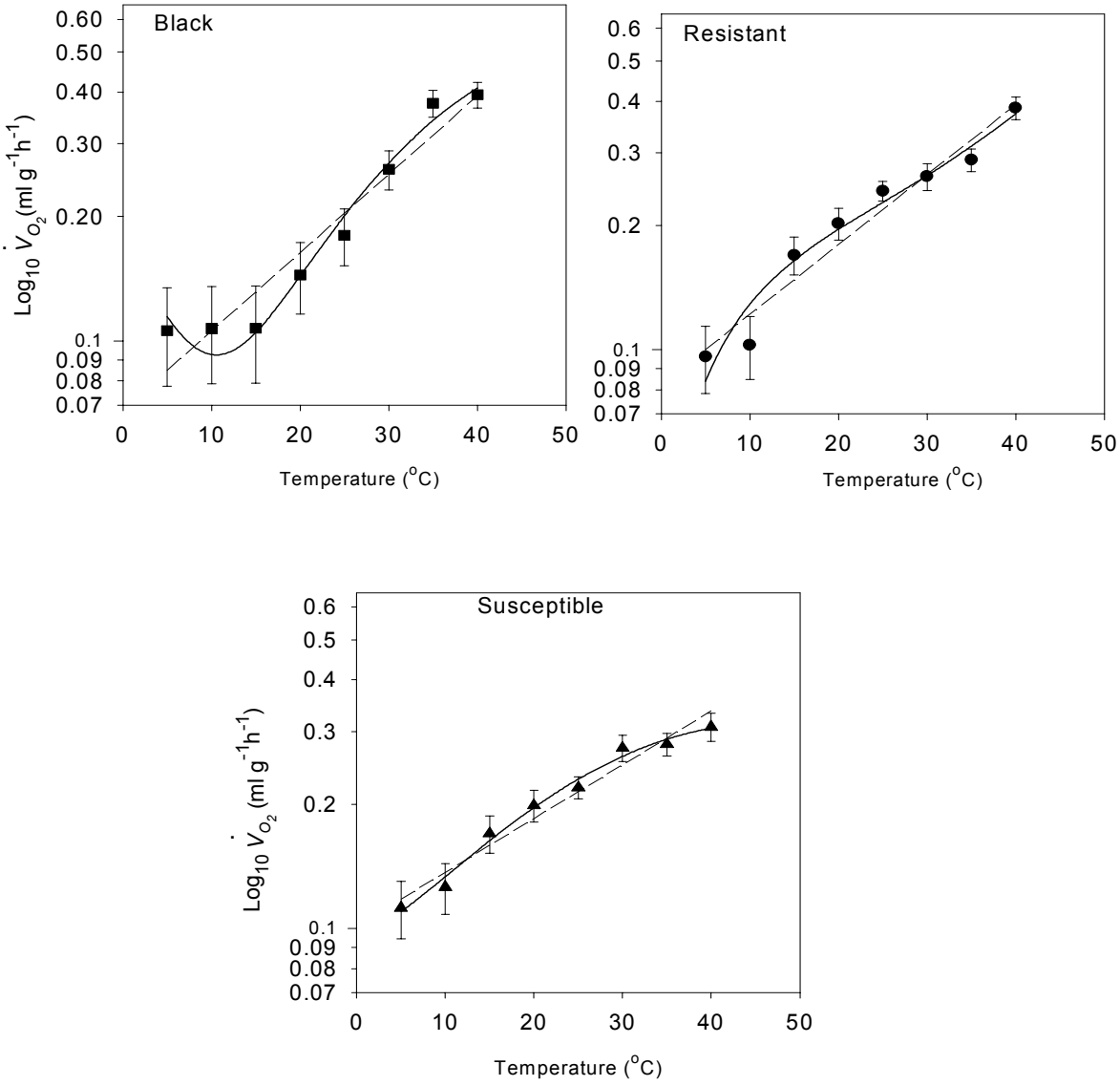

Black
Resistant
Susceptible
$\dot{V}_{O_2}$ (ml g^{-1}h^{-1})
Log$_{10}$ $\dot{V}_{O_2}$ (ml g^{-1}h^{-1})
Temperature (^{o}C)
0.60
0.50
0.40
0.30
0.20
0.1
0.09
0.08
0.07
0.6
0.5
0.4
0.3
0.2
0
10
20
30
40
50

Fig. 4.1

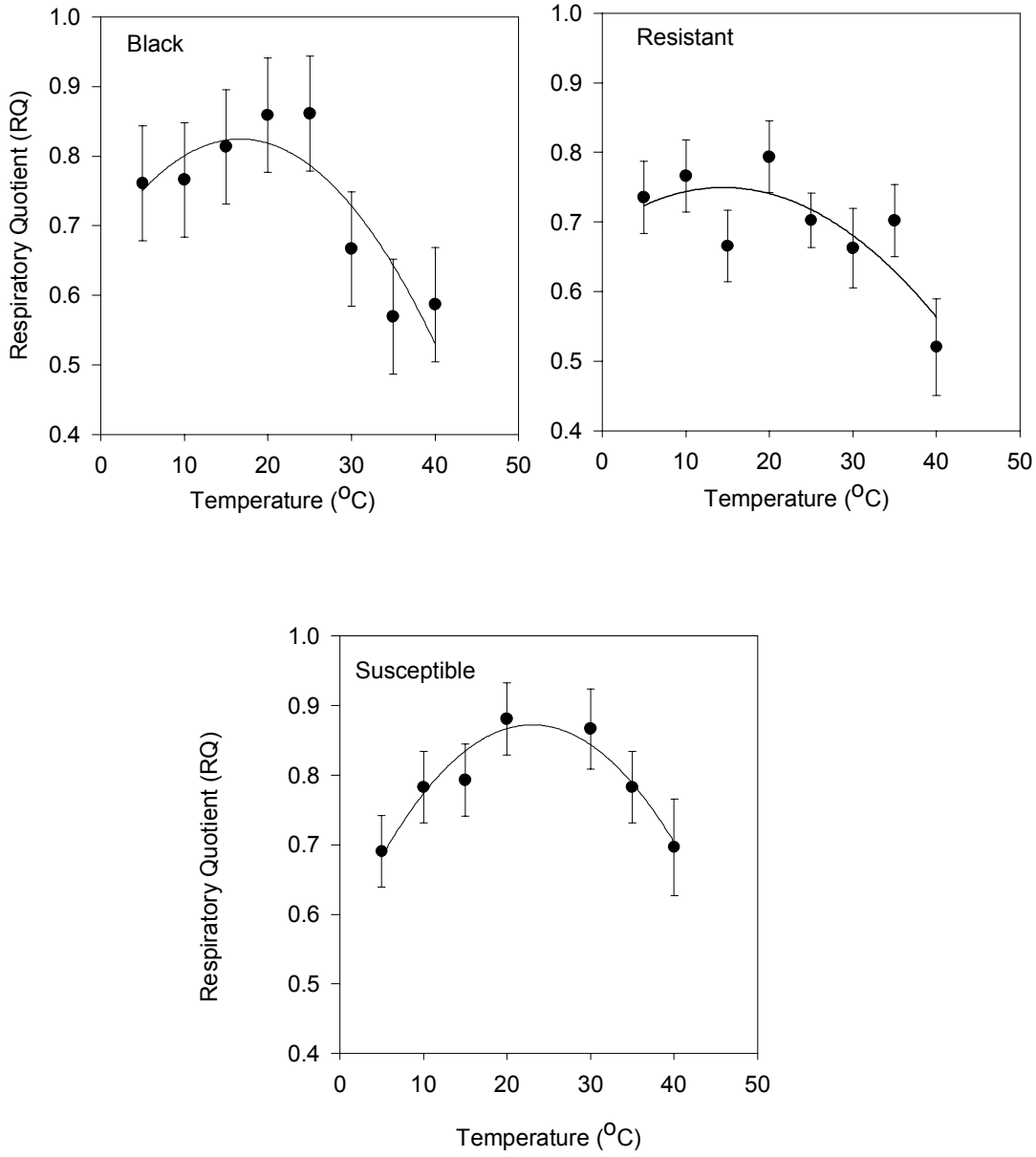

Black
Resistant
Susceptible
Respiratory Quotient (RQ)
Temperature (°C)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0
10
20
30
40
50

Fig. 4.2

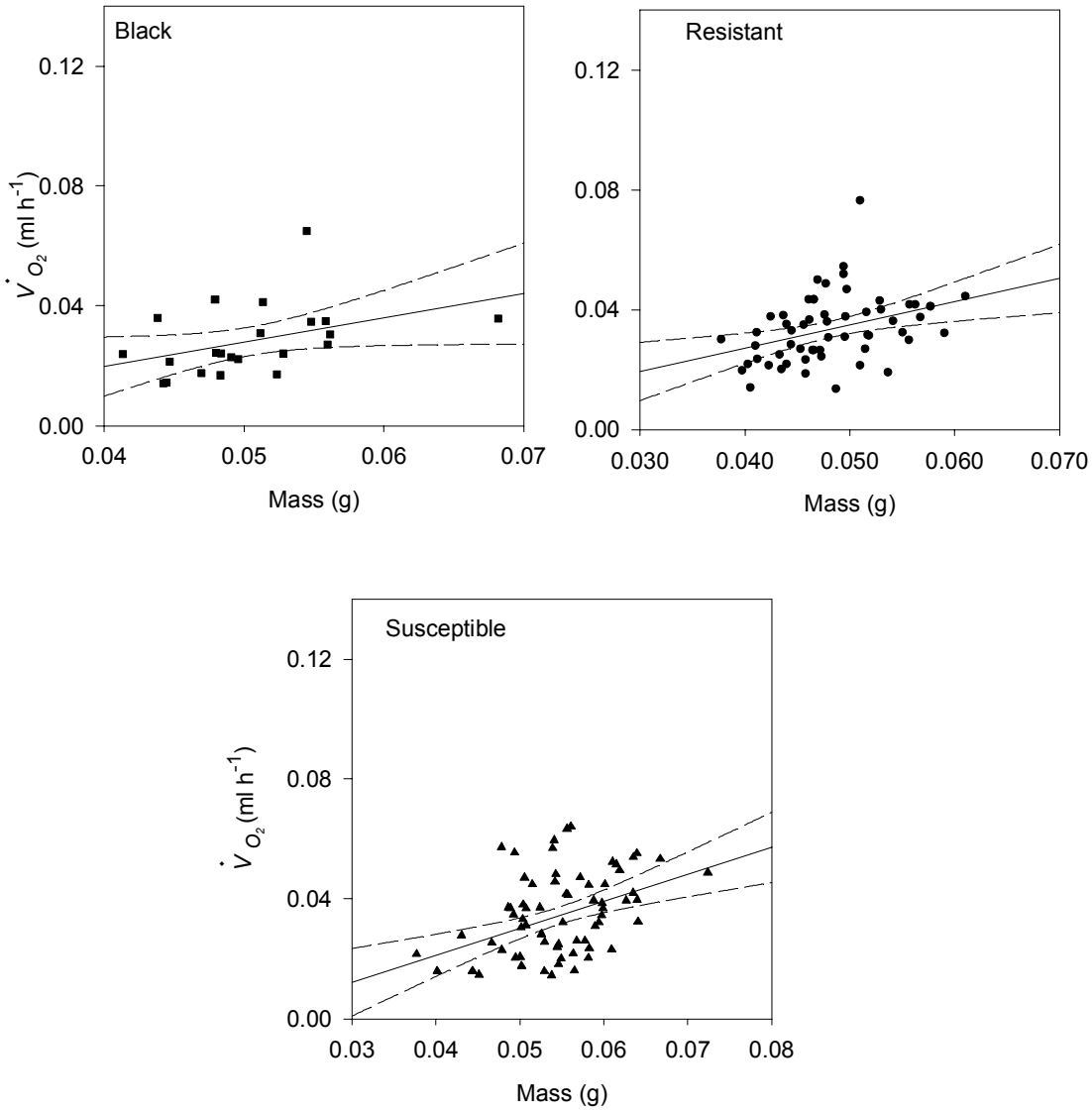

Black
Resistant
Susceptible
0.12
0.08
0.04
0.00
$\dot{V}_{O_2}$ (ml h^{-1})
0.04
0.05
0.06
0.07
0.030
0.040
0.050
0.060
0.070
0.03
0.08
Mass (g)

Fig. 4.3

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CHAPTER 5: DISCONTINUOUS CARBON DIOXIDE RELEASE IN THE GERMAN COCKROACH *BLATELLA GERMANICA* (DICTYOPTERA: BLATELLIDAE) AND ITS EFFECTS ON RESPIRATORY TRANSPIRATION

INTRODUCTION

It is becoming increasingly clear that gas exchange in many quiescent adult terrestrial insects is discontinuous (see Kestler, 1985; Slama, 1988; Lighton, 1994). A typical discontinuous gas exchange cycle (DGC) begins with a closed-spiracle phase, when little external gas exchange takes place. This is followed by a fluttering-spiracle phase where the spiracles open and close rapidly enabling gases to pass through by convection and diffusion, and finally an open-spiracle phase during which accumulated CO_2 escapes from the tracheal system to the surrounding environment (Lighton, 1996). In some insects, DGC is reported to conserve body water (Kestler, 1985; Hadley, 1994; Lighton, 1996), as a result of reduced respiratory water loss during the closed phase, and low water loss during the flutter phase (Lighton, 1996).

In insects that exhibit DGC, it is possible to determine the significance of the discontinuous gas exchange pattern in water conservation since water lost during the open phase (burst) (cuticular and respiratory loss) can be separated from loss during the closed and flutter phases (interburst; cuticular loss) (Hadley, 1994). Even though cuticular transpiration is greatly reduced by lipids associated with the epicuticle, the

cuticle is still considered the primary water-efflux path because of the large surface area to volume ratio of insects (Hadley, 1986, 1989). Regulation of cutaneous water loss is critical for small insects such as the German cockroach, *Blattella germanica* (L.), because of their small size (< 15 mm long) and large surface area to volume ratio.

Blattella germanica is a world wide household pest, which may harbor and transmit human disease-causing pathogens (Ramirez, 1989). Their body parts and feces are also potent allergens to sensitive people (Roberts, 1996). Pyrethroid insecticides are widely used for *B. germanica* control because of their effectiveness and low mammalian toxicity. However, control failures in some field populations have been reported as a result of the development of resistance (Cochran, 1989; Valles et al., 2000). Resistance levels generally decline in the absence of insecticide selection (Tabashnik et al., 1994; Rahardja and Whalon, 1995), and a decrease in resistance may be associated with increased biotic fitness (Tabashnik et al., 1994). Fitness can be observed as changes in survival rate, egg hatch, weight and metabolic rate (Groeters et al., 1993; Idris and Grafius, 1996; 1997; Dingha et al., 2004).

In cockroaches, the DGC pattern has been reported and described at 20^{o}C in the American cockroach, *Periplaneta americana* (L.) (Kestler, 1985, 1991; Machin et al., 1991), and *Perisphaeria* sp. (Marais and Chown, 2003). A cyclic O_2 consumption pattern was recorded from the tropical cockroach, *Blaberus giganteus* (L.) at 26.6^{o}C (Bartholomew and Lighton, 1985). The aim of this study was to describe the respiratory gas exchange patterns at different temperatures, investigate the effects of DGC on water loss, and determine if there are differences in the DGC characteristics between insecticide resistant and susceptible strains of *B. germanica*. To accomplish this, I conducted

experiments to test two major hypotheses. First, I hypothesized that *B. germanica* would show a pronounced DGC only at lower temperatures and continuous cycling of CO_2 release at higher temperatures. Since increased temperature generally causes the metabolic rate of ectotherm to rise, the frequency of spiracular opening would increase at higher temperatures (Chappell and Rogowitz, 2000). Therefore DGCs would be more pronounced in insects at lower temperatures and cyclic at higher temperatures. For example, in some adult insects, such as the eucalyptus-boring beetle, *Phorocantha spp*. (Chappell and Rogowitz, 2000), the California grasshopper, *Melanoplus sanguinipes* (Rourke, 2000), *P. americana* (Kestler, 1985, 1991; Machin et al., 1991) and *Perisphaeria* sp. (Marais and Chown, 2003), DGC was not exhibited at higher temperatures, instead it was observed only at lower temperatures. Second, I hypothesized that genetically resistant *B. germanica* not exposed to insecticide for several generations would have similar metabolic rates and DGC characteristics as a susceptible strain. In addition, I hypothesized there would be no significant difference in water loss between the two strains.

MATERIALS AND METHODS

Cockroach strains

Two B. germanica strains were used in this study. ACY (American Cyanamid Co., Clifton, NY), is an insecticide susceptible strain that has been reared in the laboratory without exposure to insecticide for >40 years. The second is Apyr-R (Alabama, pyrethroid resistant), a resistant strain collected from an infested kitchen in Opelika, Lee County, Alabama, U.S.A in 1999 after control failures with permethrin and deltamethrin. This strain was subsequently selected with permethrin and deltamethrin for

several generations prior to this study (Wei et al., 2001; Pridgeon et al., 2002). Levels of resistance to permethrin and deltamethrin in Apyr-R were 97- and 480- fold, respectively, compared with the susceptible strain (Wei et al., 2001; Pridgeon et al., 2002). All cockroaches were reared at 25±2°C and 50±10% RH, with photoperiod of 12L: 12D. Dry dog chow and water were supplied *ad libitum*.

Respirometry and metabolic rate measurement

I recorded patterns of CO_2 emission of individual adult male *B. germanica* at 5, 10, 15, 20, 25, 30, and 35°C using flow through respirometry. Males were selected to avoid complications arising from the metabolic demands of oogenesis in females. To avoid stress (Kestler, 1991; Machin et al., 1991), cockroaches were allowed to crawl into a 3 cm by 1.0 cm transparent Tygon® tubing respirometer chamber which was then sealed at both ends with a rubber stopper and connected to the CO_2 and H_2O analyzer. Cockroaches were acclimated in the respirometry chamber for ~1h before recordings were initiated. This allowed for acclimation to the initial temperature and predesiccation to remove any surface moisture. The respirometer was housed in a Sable Systems (Henderson, NV, USA) PT-1 Peltier-effect temperature-controlled cabinet at various temperatures. Outside air was scrubbed of CO_2 and H_2O using a Whatman purge gas Generator (Whatman, Inc., Haverhill MA, USA), drawn through a computer-controlled base lining system, a Li–Cor CO_2 and H_2O analyzer (LI-6262; LiCor Inc., Lincoln, Nebraska, USA), and a Side-Track mass flow meter (Sierra Instruments Inc., Monterey, CA, USA) with a pump (Gast Mfg. Corp., Benton Harbor, MI, USA) at a flow rate of 100 ml min^{-1} at STP. The gas sample passed through the system and data from the CO_2 and

H_2O analyzer were recorded using DATACAN V (Version 5.2; Sable Systems, Herderson, NV, USA) software. The CO_2 analyzer was calibrated with 94.9 ppm span gas (Air Products, Inc.). The occurrence of a DGC in *B. germanica* was most frequent (62.8%) at 10°C, therefore, CO_2 emission and H_2O loss were recorded simultaneously at this temperature for 2-3 h and cockroaches were weighed after each run. However, not every cockroach that was placed in the respirometry system at 10°C performed a DGC that could be analyzed completely. Recordings in which DGC was interrupted were not included in the analysis. All calculations of rate and duration of CO_2 release and H_2O loss were carried out using DATACAN V software. CO_2 and H_2O recordings were baseline-corrected and converted to $\dot{V}_{CO_2}$ ml h^{-1} and mg h^{-1} for CO_2 emission and water loss, respectively. For each individual cockroach, mean $\dot{V}_{CO_2}$ emission and durations were measured for each of the DGC phases. To minimize handling stress that may cause changes in the $\dot{V}_{CO_2}$, mass loss of animals used within the experiments was calculated by subtracting the body mass after a run from the mean body mass of 160 adult male *B. germanica* of both strains that were not used in the experiments; estimates of water loss during the run were consistent with flow-through measurements.

Data analysis

Analysis of covariance (ANCOVA) was used to estimate the effects of metabolic rate and body mass for each strain of *B. germanica* at 10°C. Regression analysis was performed if there was a significant effect of mass to estimate the slope of Log $\dot{V}_{CO_2}$ and Log mass relationship. A modified *T*-test was used to test if slope was significantly

different than 1 (Sokal and Rohlf, 1981). In measuring metabolic rate, I examined recordings from 12 susceptible and 19 resistant cockroaches. Eight to 10 DGCs were analyzed for each individual cockroach. For water loss measurements, recordings from 5 cockroaches of both strains were examined and 8 to 10 DGCs were analyzed for each individual cockroach. Because each DGC represented a replicate that may change over the period of the recording, comparison within and between strains over time was analyzed using repeated measures analysis of variance (ANOVA). The test of fixed effects (PROC Mixed Procedure, ANOVA; Khattree and Naik, 1996, SAS Institute, 1996) was used to determine the effect of strain, DGC by strain interaction and the effect of the sequence of cycles on $\dot{V}_{CO_2}$ and duration. The DGC characteristics including interburst and burst phase durations, $\dot{V}_{CO_2}$ (ml h^{-1}), H_2O loss (mg h^{-1}), and overall strain effects were analyzed using the general linear model procedure (Proc GLM, ANOVA, SAS Institute, 1996). Mean separation was carried out using the Ryan-Einot Gabriel-Welsch Multiple Range test (REGWQ) that controls both type I and type II error. The significance level was set at $P<0.05$. In view of the lack of a distinguishable closed and flutter phase during the exhibition of most DGCs in these cockroaches, I used a statistical technique that involved regressing water-loss rate against CO_2 release for each cockroach (Gibbs and Johnson, 2004; Johnson and Gibbs, 2004). Water loss rate (mg h^{-1}) at the intercept, where CO_2 release (ml h^{-1}) equals zero, was assumed to represent cuticular transpiration. Respiratory water loss was calculated as the difference between total water loss and cuticular water loss. Cuticular and respiratory water loss (mg) were expressed as percentages of total water loss. Water loss through the cuticle or cuticular permeability

(CP) was calculated as the micrograms of water lost during the entire recording per unit body surface area (cm^2) per unit time (h) per unit saturation deficit (mmHg). Surface area was estimated for each cockroach strain using Meeh's formula (Meeh, 1897).

RESULTS

Carbon dioxide emission patterns of both pyrethroid resistant and susceptible strains of B. germanica varied greatly with temperature. At 5°C there were no recordings of an interburst or burst phase for >1 h and the CO_2 emission pattern remained acyclic throughout this time. At 10°C (Fig. 5.1), 15 and 20°C (Fig. 5.2), the pattern of CO_2 emission was discontinuous. In most DGCs at 10°C, CO_2 release never dropped to zero during interburst phase in both strains of *B. germanica* (Fig. 5.2, 5.3, and 5.4). This suggests that small amounts of this gas diffuse through incompletely closed spiracles during this time. DGC frequency increased at 25 and 30°C and became cyclic at 35°C (Fig. 5.2 and 5.3). The probability that *B. germanica* would respire discontinuously varied with temperature. At 10°C, ~ 62.8% of individuals showed a pronounced DGC (n= 49 of 78); 28% at 15°C (n=7 of 25); 18% at 20°C (n=4 of 22); 20% at 25°C (n= 4 of 20); 10% at 30 and 35°C (n=1of 10 for each). Therefore, measurement of metabolic rate and water loss was carried out at 10°C. I observed that cockroaches would only exhibit DGC when the temperature in the respirometer with the cockroach was decreased gradually from ambient to the required temperature. If the temperature in the respirometer was already set prior to recording, no DGC was observed and the pattern was always cyclic. Ninety-five percent of the time, it took ~1 h for cockroaches in the respirometer chamber at 10°C to begin exhibiting DGC (Fig. 5.4). Out of the 78 DGC recordings obtained at 10°C, not every recording could be analyzed completely. Recordings in which DGC was

interrupted were not included in the analysis. Therefore, overall metabolic rate $\dot{V}_{CO_2}$ (ml h^{-1}) measured as CO_2 release over the entire recording was calculated from 12 susceptible and 19 resistant individual cockroaches. From Table 5.1 the rate of CO_2 emission between both strains was not significantly different ($F_{1,29}$ =0.24, P>0.63). Metabolic rate scaled allometrically with body mass with a slope of 1.22±0.27; this value was not significantly different than 1. The characteristics of the DGC shown in Table 5.1 were calculated from a total of 96 DGCs from 12 susceptible individuals and 171 DGCs from 19 resistant individuals all at 10°C. The rate of CO_2 emission ($\dot{V}_{CO_2}$ ml h^{-1}) in each phase is the volume of CO_2 emitted during that phase, obtained by calculating the area under the CO_2 peak and then divided by the phase duration. The phase duration is the time taken for CO_2 to be emitted during that phase and the period is the duration of one DGC (i.e., burst + interburst phase). Interburst and burst $\dot{V}_{CO_2}$ (ml h^{-1}) were not significantly different between the two strains (Table 5.1 and 5.2.). The variability over time in CO_2 emission during interburst and burst phases was not significantly different from cycle to cycle or between strains (Table 5.2). However, there were significant differences in the durations of the interburst and burst phases between the two strains (Table 5.3). The susceptible strain had significantly longer interburst and burst phase durations during a complete DGC than the resistant strain (Table 5.1). This results in a DGC of significantly longer duration (13.89±0.44 min) than in the resistant strain (11.23±0.26 min). Also, each phase (interburst or burst) accounted for ~50% of the DGC cycle duration in the susceptible strain (Table 5.1). In contrast, in the insecticide resistant strain the interburst phase accounted for 43.5% and the burst phase 56.5% of the cycle duration (Table 5.1).

However, in both strains, ~90% of total CO_2 release during a DGC occurred in the burst phase (Table 5.1). The duration of the interburst phase of both pyrethroid resistant and susceptible strains increased with increasing DGC duration (Fig. 5.5A and B). However, the duration of the interburst phase in the resistant strain decreased as $\dot{V}_{CO_2}$ decreased, but increased in the susceptible strain (Fig. 5.6A and B). Mean cockroach body mass differed significantly between strains (Table 5.1). Mean body mass was calculated from 160 adult male *B. germanica* of both strains. The close synchrony between CO_2 bursts and bursts of water loss provides a means of separating and quantifying cuticular and respiratory water loss (Fig. 5.7). Respiratory water loss is presumed to be minimal during interburst periods since a reduction in continuous spiraclar opening would reduce water loss. Cuticular water loss was significantly greater in the susceptible strain compared with the resistant (Fig. 5.8). Also, total water loss (ml h^{-1}) was significantly greater in the susceptible strain (Table 5.4). In both strains cuticular transpiration always accounted for >95% of the total water loss (Table 5.4). The overall water loss (mg h^{-1}) was not significantly different ($F_{1, 8}$=0.31, P>0.59) between strains. Cuticular permeability (CP) values using Meeh (1897) surface area model estimates were significantly different (P<0.05) between strains. The CP was 2.26 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the resistant strain and 3.42 μg cm^{-2} h^{-1} $mmHg^{-1}$ for the susceptible strain.

DISCUSSION

Variation of metabolic rate with temperature has long been known in insects. The effect of temperature however, on the gas exchange pattern has not been examined in most adult insects. In the majority (62.8%) of pyrethroid resistant and susceptible *B. germanica* tested in this study, the discontinuous CO_2 emission pattern was observed at 10^{o}C and in a few individuals at 15 and 20^{o}C. Increase in temperature (25 and 30^{o}C) was accompanied by an increase in the frequency of the DGC pattern. At 35^{o}C, CO_2 emission was cyclic even though relatively few individuals showed discontinuous CO_2 release at this higher temperature. Chappell and Rogowitz (2000) reported similar results in the Eucalyptus-boring beetles, *Phorocantha* sp. which exhibited DGC at 10 and 20^{o}C, but the pattern changed to cyclic CO_2 emission at temperatures $\geq$30^{o}C. However, a few beetles exhibited DGC at these high temperatures. These findings indicate that within a given insect species and when insects are inactive, the majority of individuals exhibit DGC over a certain temperature range above which only a few individuals would exhibit DGC. This inconsistency in displaying a DGC could be due to genetic and/or physiological variation in any given population. However, in most published studies, the proportion of individuals that exhibit the DGC is not reported.

The DGCs of both pyrethroid resistant and susceptible strains of *B. germanica* were exhibited only at low to ambient temperatures when cockroaches were quiet and undisturbed. These are similar conditions in which discontinuous CO_2 emission was observed in *R. guttata* (Hadley and Quinlan, 1993), *P. americana* (Kestler, 1991), and *Perisphaeria* sp. (Marais and Chown, 2003). A probable cause could be that at lower temperatures, metabolism is reduced and therefore CO_2 production declines. Thus, the

spiracles remain closed longer and are only opened when the concentration of CO_2 within the tracheal system has increased to a certain threshold. Whereas at higher temperatures with increased metabolism, more CO_2 is released from tissues per unit time (Schneiderman, 1960). One implication is that cycle frequency will increase with increased metabolism, and gas exchange will become continuous at higher temperatures as illustrated in Fig. 5.3.

The discontinuous CO_2 emission pattern in both strains of *B. germanica* differed from the classic ‘‘three phase’’ pattern first described in lepidopteran pupae (Schneiderman, 1960), and that seen in *P. americana* and *Perisphaeria* sp. (Kestler, 1991; Marais and Chown, 2003). The interbust phase could not be clearly differentiated into closed and flutter phases and $\dot{V}_{CO_2}$ never fell to zero in most (~60%) of the DGCs. This is not an unusual pattern as similar observations were also noted in the Eastern lubber grasshopper, *Romalea guttata* (Hadley and Quinlan, 1993), the eucalyptus-boring beetle, *Phorocantha* spp. (Chappell and Rogowitz, 2000), and in the dampwood termite, *Zootermopsis nevadensis* where it was referred to as an acyclic CO_2 emission pattern (Shelton and Appel, 2000). One possibility for this type of pattern is that these insects may be using independent spiracles during respiration in which case some spiracles are kept open while others are closed or occasionally all spiracles are closed. In addition, it is possible that it is more difficult for insects to precisely coordinate the opening and closing of many spiracles. Adult *B. germanica* have 4 thoracic and 16 abdominal spiracles (Haber, 1962) compared with as few as 4 in many adult ants. Unfortunately, we could not observe spiracular behavior directly during our measurements. Therefore, I do not know whether all the spiracles remained opened at all times.

Change in respiratory pattern from discontinuous to continuous CO_2 emission as a result of a stressor has been documented. For example, in the presence of insecticides (Kestler, 1991), toxic plant extracts (Sibul et al., 2003; Kuusik et al., 2001; Harak et al., 1999) and handling (Harak et al., 1998), the pattern of CO_2 release changed from DGC to continuous emission. In this study, I observed that when the temperature in the respirometer decreased gradually from room temperature to 10°C, the pattern of CO_2 emission was discontinuous. However, when *B. germanica* was placed directly into a chamber already at 10°C, the CO_2 emission pattern was continuous. This difference in CO_2 emission pattern could be a response to cold (stressor).

In this study, the metabolic rate between genetically resistant *B. germanica* not exposed to insecticide after several generations was not significantly different from the susceptible strain. Similarly, Hostetler et al. (1994) using closed system respirometry at 26°C, reported no significant difference in metabolic rate between pyrethroid resistant and susceptible *B. germanica.* Resistant alleles have been shown to exert negative effects on fitness, such as reduced mating (Groeters et al., 1993), decreased weight (Brewer and Trumble, 1991; Hostetler and Brenner, 1994; Dingha et al., 2004), reduced egg hatch (Ross, 1991) and increased metabolic rate (Dingha et al., 2004). In the absence of selection, resistance has been reported to decline, for example in *B. thuringiensis* resistant diamondback moth, *Plutella xylostella* (Tabashnik et al., 1994) and the Colorado potato beetle, *Leptinotarsa decemlineata* (Rahardja and Whalon, 1995). Also in other pyrethroid resistant *B. germanica* resistance declined from 140 to 1.6 fold in the absence of selection pressure after 15 generations (Cochran, 1993). This reversion is likely associated with increased fitness (Tabashnik, 1994). For example, susceptible and resistant *S. exigua*

pupae whose larvae were not exposed to Cry1C *B. thuringiensis* had similar metabolic rates however, this was significantly different from pupae whose larvae were exposed continuously to toxin (Dingha et al., 2004). The mechanisms of resistance to pyrethriod in *B. germanica* include P450 monooxygenase, hydrolase, and altered sodium channels (Wei et al., 2001, Pridgeon et al., 2002). The resistant strain of *B. germanic*a may have detoxification mechanisms that would increase metabolic rates but requires the presence of the insecticide to induce accelerated production of the detoxifying enzymes. Furthermore, several studies have documented that production of detoxifying enzymes increase in the presence of pesticides (e.g., Terriere, 1983).

At 25^{o}C, the metabolic rates for both strains was 0.034 ml h^{-1}, calculated using Q_{10} values of 1.19 and 1.15 for the resistant and susceptible *B. germanica,* respectively (Dingha et al. unpublished). The metabolic rate of other cockroaches such as, *Perisphaeria* sp. (0.315g) found at high altitudes (950m above sea level) was 0.0207 ml h^{-1} at 20^{o}C (Marais and Chown, 2003). The oxygen consumption rate of *B. giganteus* (4.33g) measured at the end of a 6-10 min run was 0.693 ml h^{-1} at 26.6^{o}C (Bartholomew and Lighton, 1985). Similar values (0.0316 and 0.034 ml h^{-1}) were reported for pyrethroid resistant (0.048g) and susceptible (0.043g) *B. germanica*, respectively at 26^{o}C (Hostetler et al., 1994).

Generally, during a DGC the closed phase begins at the end of the last open phase, and lasts until endotracheal O_2 concentration reaches a critical threshold (Levy and Schneiderman, 1966). Once this threshold is reached, the flutter phase is initiated, and during this time, CO_2 accumulates in the hemolymph and continues to accumulate until the open phase is triggered. In both pyrethroid resistant and susceptible *B. germanica* the

interburst and burst phase durations were linearly related with the duration of the DGC (Fig. 5.5A and B). It is therefore possible that both set points influence the duration of the interburst phase. In addition, local concentrations of CO_2 and O_2 in adult insects, including cockroaches, affect spiracular movements (Harrison et al., 1995). Even though we did not measure internal changes in CO_2 and O_2 concentrations, it is possible that different CO_2 and O_2 thresholds trigger spiracular opening in these two physiologically distinct strains (Schneiderman and Williams, 1955; Harrison et al., 1995).

Terrestrial insects generally lose water through several pathways, such as the spiracles, cuticle, oral, and anal openings. The importance of spiracular fluttering in reducing water loss has been emphasized (Miller, 1981; Lighton and Garrigan 1995). Insects must be able to regulate spiracular opening when challenged with conditions that could result in water loss. Generally, in such situations the burst phase (high respiratory water loss) of the DGC should be short relative to the interburst phase (low respiratory water loss). Only a few studies have actually measured respiratory water loss during bouts of CO_2 emission and compared these values to rates of total water loss over the same period. Kestler (1978, 1985), measured respiratory transpiration in *P. americana* during DV cycle by recording changes in body mass with an extremely sensitive electronic balance and found the rate of mass (water) loss to be lowest during the interburst phase of the cycle, but increased at the start of ventilation. Machin et al. (1991) also reported that the mean water loss of *P. americana* during the burst phase was higher (87%) than the rate during the interburst phase. The susceptible strain has longer interburst and open phase durations than the resistant strain (Table 1). The duration of the burst phase is less than half and the interburst phase is greater than half the duration of a

complete gas exchange cycle. In the resistant strain the duration of the burst phase is greater than half and the interburst phase is less than half the duration of a complete gas exchange cycle. The long interburst phase of the susceptible strain was not important in reducing water loss because more water was lost during this time in comparison to the resistant strain. However, long flutter periods ≥23 min appear to be important in reducing water loss in arid adapted arthropods, such as the ant *Cataglyphis* sp. (Lighton and Wehner, 1993), and a trogid beetle (Bosch et al., 2000) where the need to conserve water is great.

In the resistant strain, respiratory transpiration was responsible for 3.4% of total water loss, while in the susceptible strain respiratory transpiration accounts for 4.4% of total water loss with cuticular transpiration accounting for the bulk of the water loss in both strains. Despite the high cuticular water loss in both strains (>95%) the susceptible strain lost significantly more water in a DGC than the resistant strain. This could be due to the long duration of the interburst phase compared with that of the resistant strain (Table 5.1). In *R. guttata*, cuticular respiration accounted for 97% of total water loss (Quinlan and Hadley, 1993), in *Taeniopoda eques* 95.2% (Quinlan and Hadley, 1993), 85% in *M. sanguinipes* (Rourke, 2000), and 95% in *Aphodius fossor* (Chown and Holter, 2000). In these insects respiratory transpiration is often less than 20% of the total loss. Our data for *B. germanica* are similar to these values. However, cuticular transpiration and cuticular permeability were significantly reduced in the resistant strain. Possibly selection for insecticide resistance altered the composition of the cuticle or the epicuticular lipids since decreased cuticular penetration is one of the mechanisms of resistance in the resistant *B. germanica* strain (Wei et al., 2002).

The rate of overall water loss in resistant and susceptible *B. germanica*, when expressed per unit surface area and corrected for saturation deficit, fall within the range of values (0-30 μg cm^{-2} h^{-1} $mmHg^{-1}$) exhibited by arthropods that inhabit xeric environments (Edney, 1977). If CP has a Q_{10} value of 2 (Hadley, 1994) then the CP at 30°C would be 9.04 and 13.7μg cm^{-2} h^{-1} $mmHg^{-1}$ for the resistant and susceptible strains, respectively. However, these CP values were less than half of those obtained from pyrethroid resistant (25.23μg cm^{-2} h^{-1} $mmHg^{-1}$) and susceptible (27.56μg cm^{-2} h^{-1} $mmHg^{-1}$) *B. germanica* in a still-air desiccation study (Appel, 1993). Furthermore, the CP values were not comparable to those of other cockroaches, for example *Diploptera punctata* (20.91 μg cm^{-2} h^{-1} $mmHg^{-1}$) and *Pycnoscelus surinamensis* (38.69 μg cm^{-2} h^{-1} $mmHg^{-1}$) (Appel, 1991). These differences could probably be due to the fact that Appel (1991; 1993) measured CP from dead cockroaches, which were obviously unable to regulate water loss. It is assumed that spiracular-closing and water-conserving mechanisms are intact in living insects under experimental conditions, whereas water loss in recently killed specimens is mostly by simple diffusion through open spiracles that cannot be controlled (Machin et al., 1991).

In conclusion, during discontinuous release of CO_2 in *B. germanica* >95% of water loss occurs during the interburst phase, and respiratory transpiration therefore contributed little to total water loss. Significantly longer duration of the interburst phase of the susceptible strain of *B. germanica* did not contribute to reduction of water loss. DGC tends to be replaced by continuous CO_2 emission as temperature increases and could be due to increased metabolism associated with increased temperature. Moreover, the DGC was only observed at lower temperatures and when the cockroaches were

undisturbed. In the insect's natural environment, with temperature >26^{o}C (Appel, 1995), the probability that *B. germanica* would breath discontinuously is about 20% compared with about a 60% chance at 10^{o}C.

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Table 5.1. Characteristics (mean ± SE) of the discontinuous gas exchange cycle (DGC) in resistant and susceptible German cockroaches at 10°C. The sample size (n) is the number of cockroaches measured. Values were calculated from 171 DGCs from 19 resistant and 96 DGCs from 12 susceptible *B. germanica*. Means within rows followed by the same letter are not significantly different at $P \leq 0.05$, REGWQ test.

	Strain	
	Resistant	Susceptible
Mass (mg)*	48.80 ± 0.0004b	54.00 ± 0.0005a
n	19	12
Rate of CO_2 emission:		
$\dot{V}_{CO_2}$ (ml h^{-1})	0.024 ± 0.004a	0.021 ± 0.004a
Rate of burst CO_2 emission:		
$\dot{V}_{CO_2}$ (ml h^{-1})	0.021 ± 0.004a	0.019 ± 0.004a
Burst duration (min)	6.21 ± 0.134b	6.73 ± 0.173a
Burst duration (% of DGC duration)	56.50 ± 0.008a	50.82 ± 0.012b
Rate of interburst CO_2 emission:		
$\dot{V}_{CO_2}$ (ml h^{-1})	0.0029 ± 0.0005a	0.0024 ± 0.0004a
Interburst duration (min)	5.02 ± 0.18b	7.16 ± 0.37a
Interburst (% duration of DGC duration)	43.50 ± 0.008b	49.18 ± 0.12a
Discontinuous gas exchange		
Period (min)	11.23 ± 0.26b	13.89 ± 0.44a
Frequency (mHz)	1.48 ± 0.03	1.20 ± 0.04

*Initial mass calculated from 160 individuals of each strain that were not used in the respirometry experiments (see materials and methods)

Table 5.2. Sources of variation, F-statistics, degrees of freedom (numerator, denominator) and probabilities for A) interburst, B) burst phases $\dot{V}_{CO_2}$ (ml h^{-1}) of DGC in resistant and susceptible strains of *B. germanica* at 10°C. Valueswere calculated from 171 DGCs from 19 resistant and 96 DGCs from 12 susceptible *B. germanica.* A DGC is comprised of burst+interburst phases and here it indicates the variability from cycle to cycle.

	A) Interburst $\dot{V}_{CO_2}$ (ml h^{-1})			B) Burst $\dot{V}_{CO_2}$ (ml h^{-1})		
Source	F	df	P	F	df	P
Strain	2.41	1,232	0.1220	0.63	1,232	0.4268
DGC	0.61	7,232	0.7459	0.22	1,232	0.9793
DGC*Strain	0.58	7,232	0.7690	0.21	1,232	0.9841

Table 5.3. Sources of variation, F-statistics, degrees of freedom (numerator, denominator), and probabilities for A) interburst phase duration (min), B) open phase duration (min), of DGC in resistant and susceptible strains of *B. germanica* at 10°C. Values were calculated from 171 DGCs from 19 resistant and 96 DGCs from 12 susceptible *B. germanica.* A DGC is comprised of burst+interburst phases and here it indicates the variability from cycle to cycle.

	A) Interburst (min)			B) Burst (min)		
Source	F	df	P	F	df	P
Strain	33.27	1,232	0.0001	5.42	1,232	0.0208
DGC	1.76	7,232	0.0968	0.46	1,232	0.8644
DGC*Strain	0.54	7,232	0.8005	0.44	1,232	0.8778

Table 5.4. Water loss rates of the discontinuous gas exchange cycle in pyrethroid resistant and susceptible *B. germanica* at 10°C.

	Strain	
Variable	Resistant	Susceptible
Cuticular water loss rate (mg h^{-1})	0.033 ± 0.005b	0.054 ± 0.007a
Respiratory water loss rate (mg h^{-1})	0.002 ±0.001a	0.002 ± 0.001a
Total water loss (mg h^{-1})	0.035 ± 0.006b	0.056 ± 0.007a
% Respiratory water loss	3.4 ±1.90a	4.4 ± 2.15a

Note: Water loss values were calculated from ~ 55-60 DGCs from 5 cockroaches per strain

[a] Means within rows followed by the same letter are not significantly different at $P<0.05$, REGWQ test

FIGURE LEGENDS

Fig. 5.1. Typical recording of DGC in (a) resistant (0.045g) and (b) susceptible (0.049g) *B. germanica* measured over 1 hour at 10^{o}C.

Fig. 5.2. The effect of temperature (15, 20, and 25^{o}C) on the DGC frequency illustrated using a susceptible *B. gernanica*. Using the same cockroach, temperature was increased after every hour by 5^{o}C. The CO_2 emission pattern seen here shows DGC at all three temperatures. Note that in some DGCs the CO_2 emission never drops to zero in both strains.

Note the increase in DGC frequency as temperature increases.

Fig. 5.3. The effect of temperature (30 and 35^{o}C) on the DGC frequency illustrated using the same cockroach as in Fig. 2. With cockroach in the respirometer, temperature was increased every hour by 5^{o}C. Note that in some DGCs the CO_2 emission never drops to zero in both strains. Note the increase in DGC frequency as temperature increases, with the CO_2 emission pattern becoming cyclic at 35^{o}C.

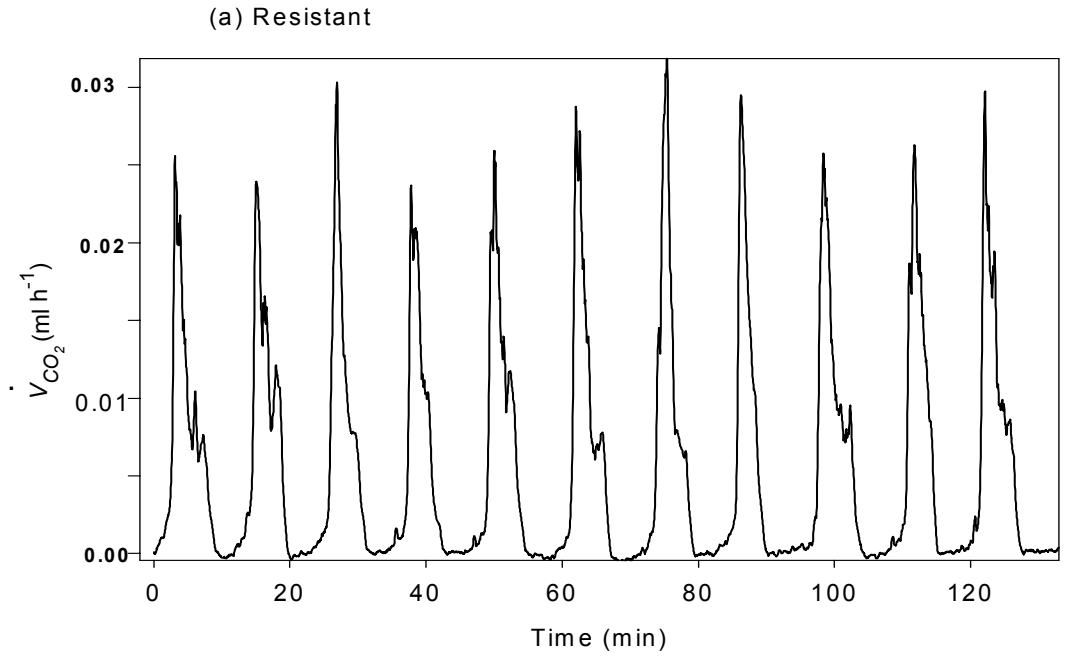
Fig. 5. 4. An illustration of the type of ventilation pattern exhibited by both pyrethroid resistant and susceptible *B. gernanica* over time as a result of acclimation to initial set temperature before they begin discontinuous gas exchange. The transition in the CO_2 emission pattern from ambient temperature to 10^{o}C can be clearly seen. It takes ~1 h before DGC is displayed. Note that in some DGCs the CO_2 emission never drops to zero in both strains.

Fig. 5.5. Relationship between duration of a complete DGC and interburst phase duration in resistant and susceptible *B. germanica. N*=12 susceptible and 19 resistant adult males, with a total of 171 DGCs in the resistant and 96 in the susceptible strains obtained at 10°C.

Fig. 5.6. The relationship between $\dot{V}_{CO_2}$ and interburst phase duration in resistant and susceptible *B. germanica. N*=12 susceptible and 19 resistant adult males, with a total of 171 DGCs in the resistant and 96 in the susceptible strains obtained at 10°C.

Fig. 5.7. A simultaneous recording of CO_2 release and H_2O loss in *B. germanica* at 10°C

Fig. 5.8. Plots of water loss (mg h^{-1}) against CO_2 emission at 10°C for (a) susceptible and (b) resistant *B. germanica*. The arrow in the lower panel denotes the *Y*-intercept of the regression, corresponding to the point at which CO_2 emission is absent and the spiracles are completely closed. The value of the *Y*-intercept thus represents cuticular water loss.


(a) Resistant
0.03
0.02
0.01
0.00
$\dot{V}_{CO_2}$ (ml h^{-1})
0
20
40
60
80
100
120
Time (min)

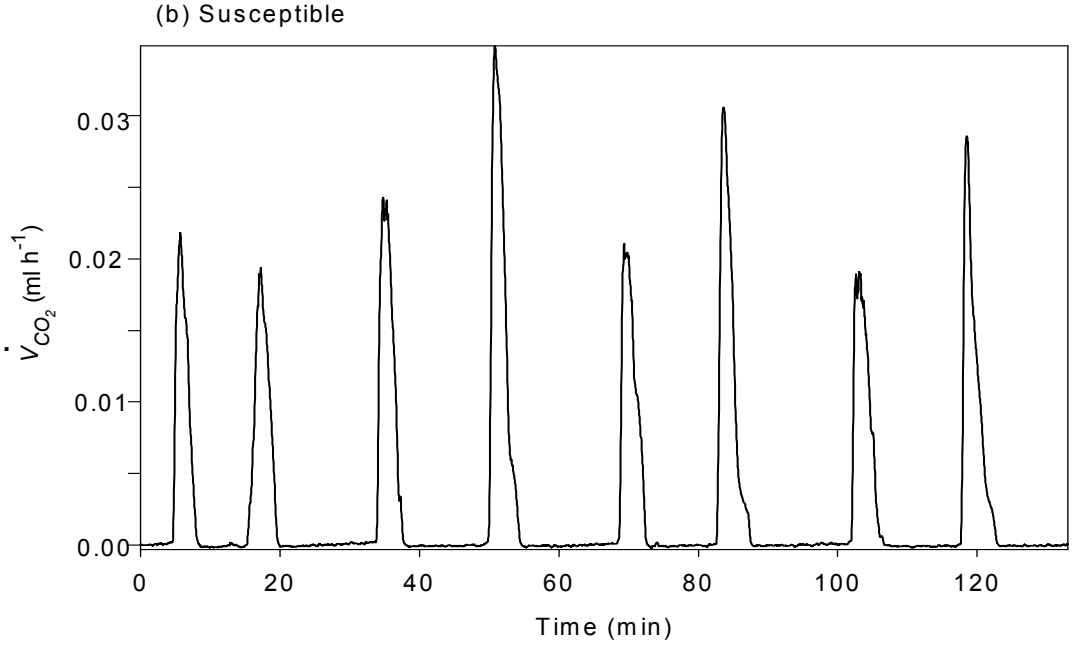

(b) Susceptible
0.03
0.02
0.01
0.00
$\dot{V}_{CO_2}$ (ml h^{-1})
0
20
40
60
80
100
120
Time (min)

Fig. 5.1

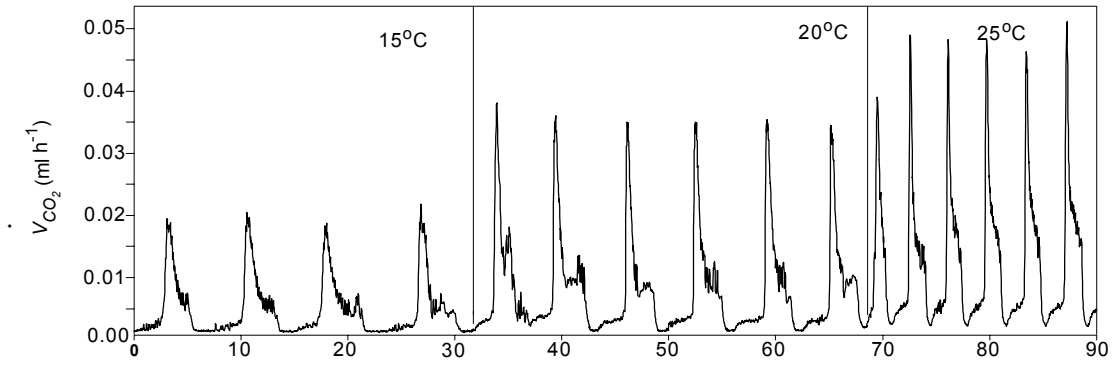

15°C
20°C
25°C
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
90
$\dot{V}_{CO_2}$ (ml h^{-1})
Time (min)

Fig. 5.2

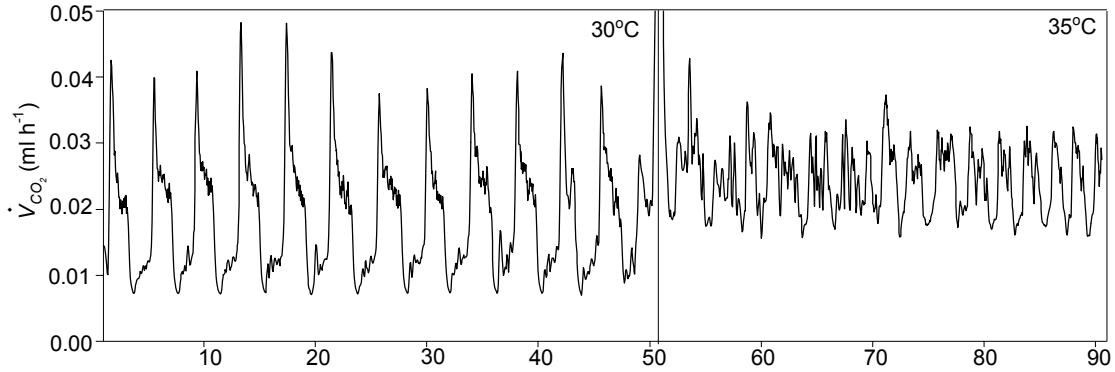

30°C
35°C
$\dot{V}_{CO_2}$ (ml h^{-1})
0.05
0.04
0.03
0.02
0.01
0.00
10
20
30
40
50
60
70
80
90
Time (min)

Fig. 5.3

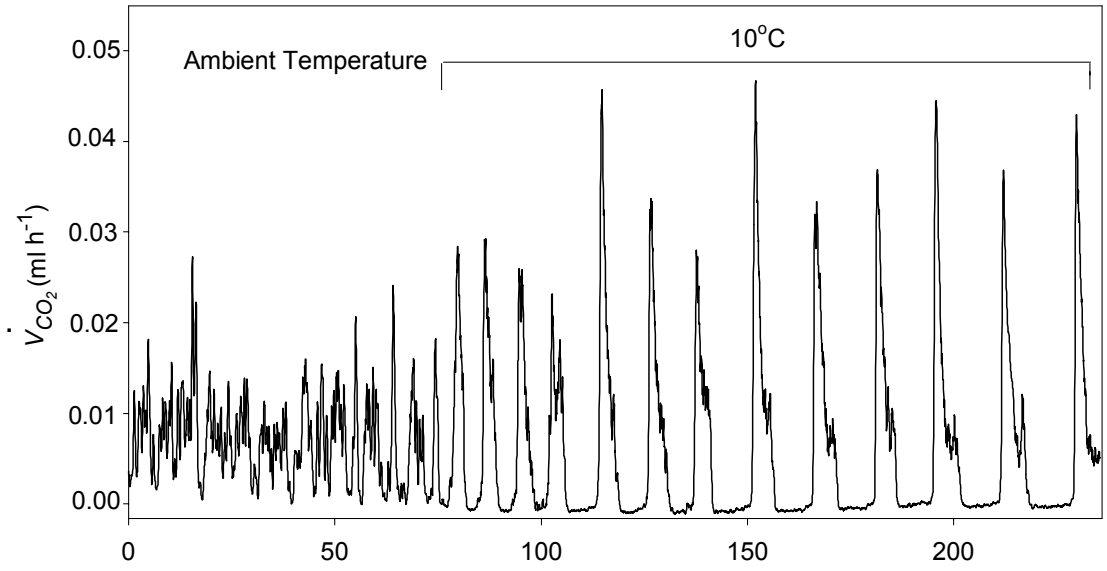
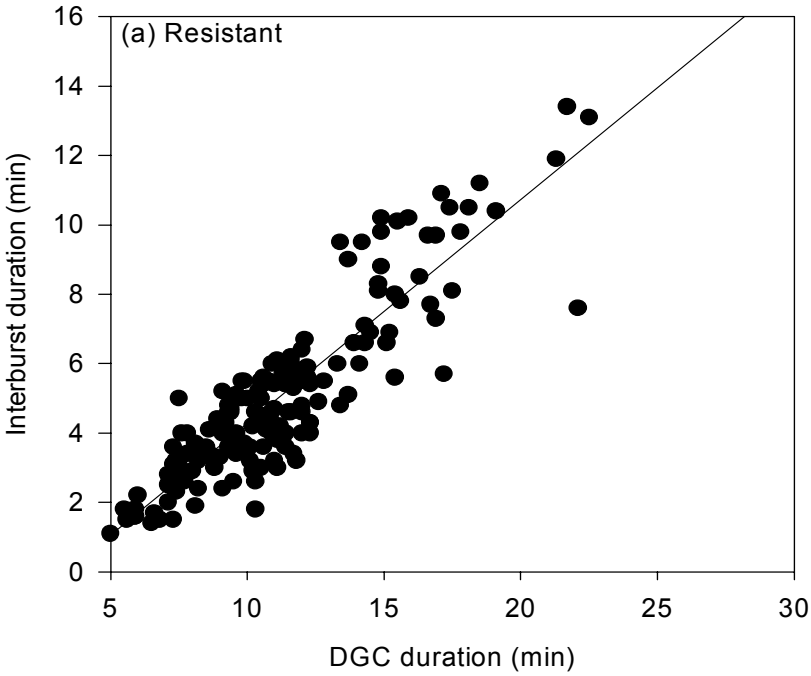

Ambient Temperature
10°C
0.05
0.04
0.03
0.02
0.01
0.00
$\dot{V}_{CO_2}$ (ml h^{-1})
0
50
100
150
200
Time (min)

Fig. 5.4


(a) Resistant
Interburst duration (min)
DGC duration (min)
0
2
4
6
8
10
12
14
16
5
10
15
20
25
30

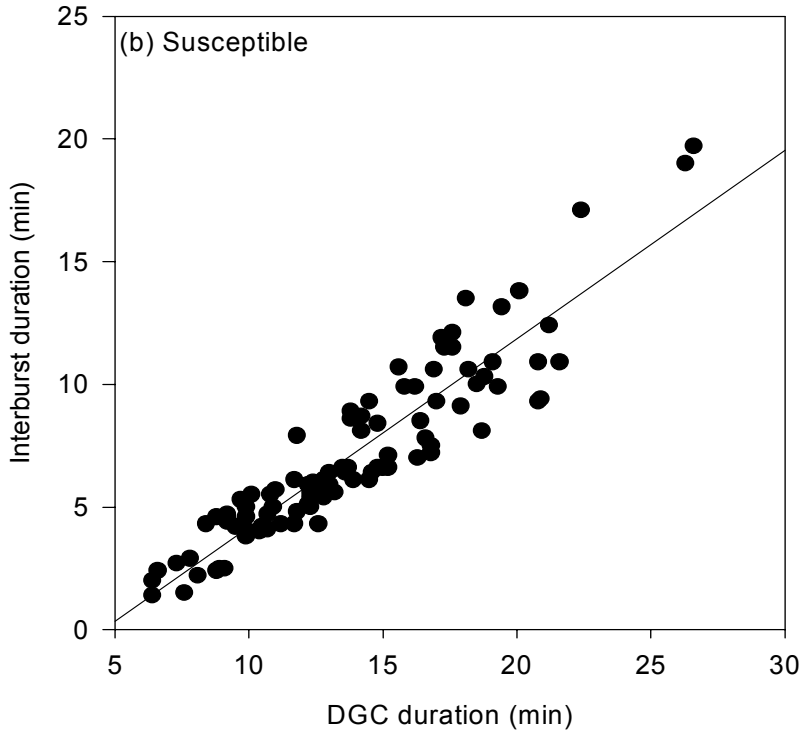

(b) Susceptible
Interburst duration (min)
DGC duration (min)
0
5
10
15
20
25
5
10
15
20
25
30

Fig. 5.5

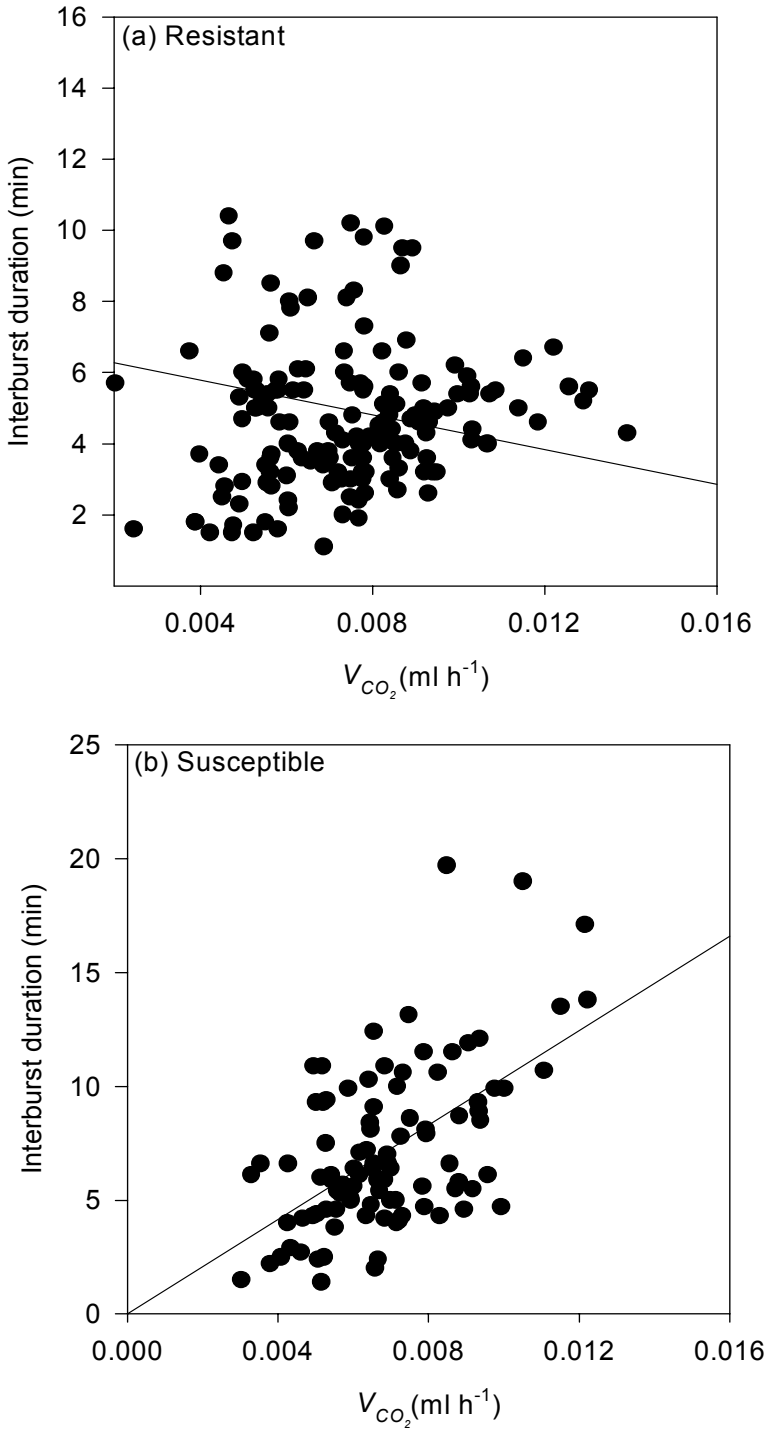

(a) Resistant
Interburst duration (min)
16
14
12
10
8
6
4
2
0.004
0.008
0.012
0.016
V_{CO_2}(ml h^{-1})
(b) Susceptible
Interburst duration (min)
25
20
15
10
5
0
0.000
0.004
0.008
0.012
0.016
V_{CO_2}(ml h^{-1})

Fig. 5.6

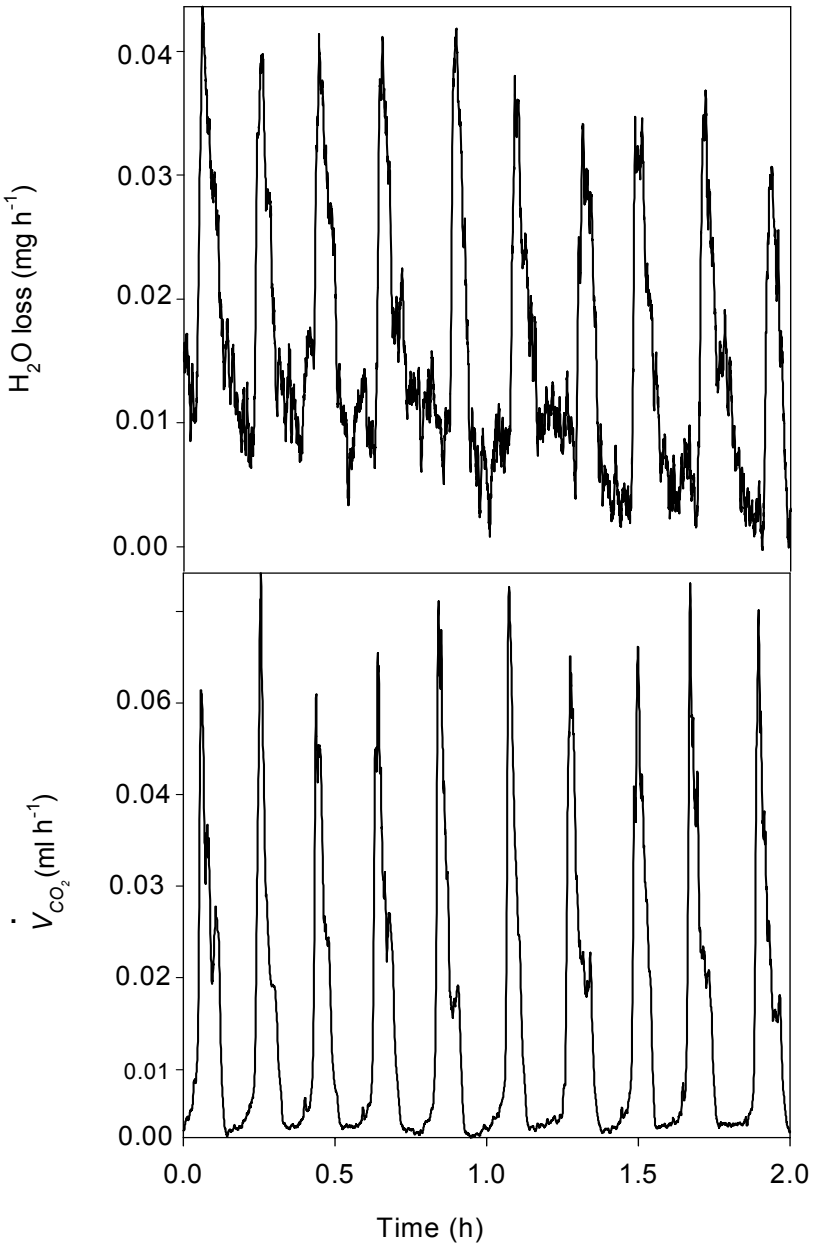
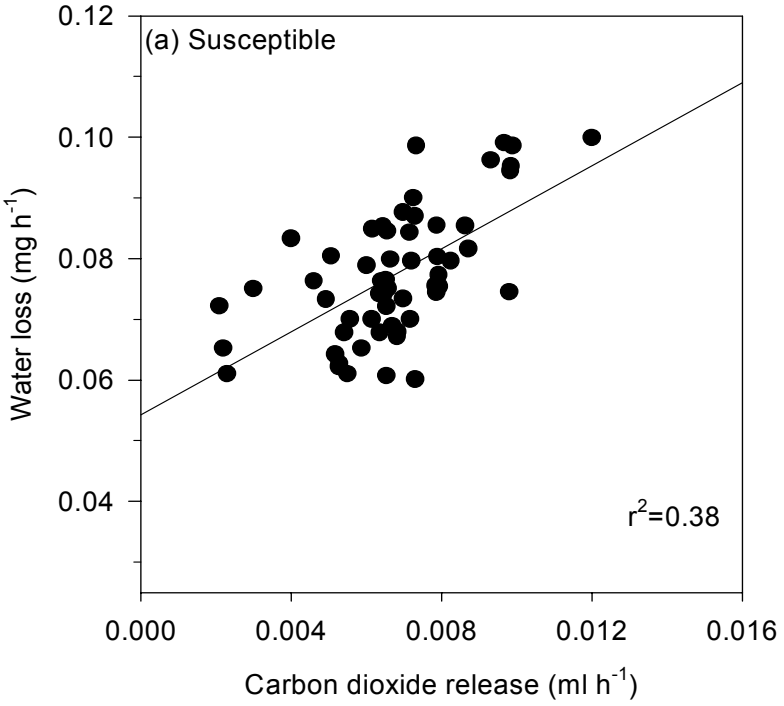

0.04
0.03
0.02
0.01
0.00
H2O loss (mg h-1)
0.06
0.04
0.03
0.02
0.01
0.00
V̇CO2 (ml h-1)
0.0
0.5
1.0
1.5
2.0
Time (h)

Fig. 5.7


(a) Susceptible
Water loss (mg h-1)
0.12
0.10
0.08
0.06
0.04
r2=0.38
0.000
0.004
0.008
0.012
0.016
Carbon dioxide release (ml h-1)

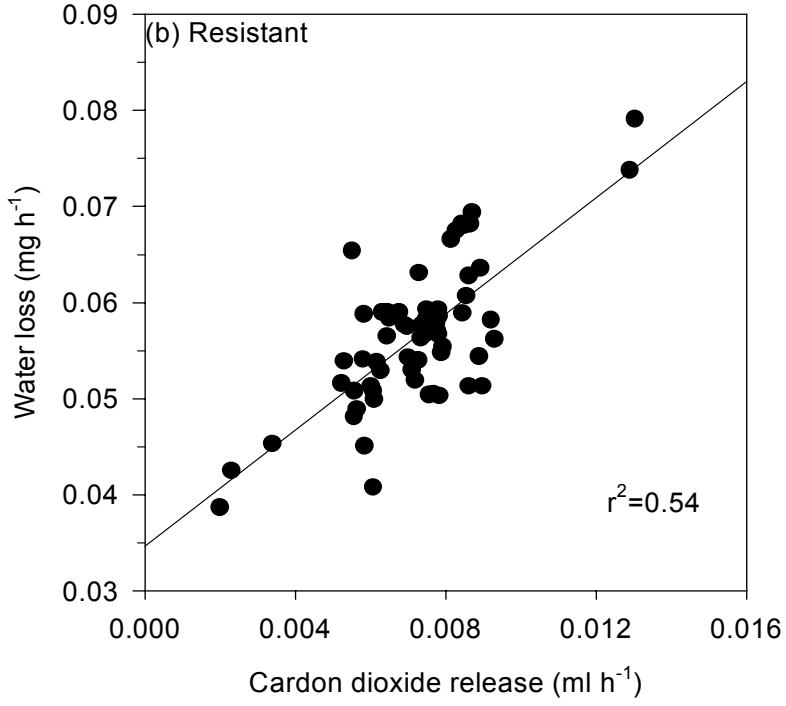

(b) Resistant
Water loss (mg h-1)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
r2=0.54
0.000
0.004
0.008
0.012
0.016
Cardon dioxide release (ml h-1)

Fig. 5.8

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