

EVALUATION OF COLOUR CHANGES, SURVIVAL RATE AND LIFE SPAN OF THE CONFUSED SAP BEETLE (*Carpophilus mutilatus*) (COLEOPTERA: NITIDULIDAE) IN DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE (CO₂)

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Abstract. This study conducted in a rearing room (RR) (300-410 ppm) and in an open roof ventilation greenhouse system (ORVS) (800-950 ppm). No changes observed on *Carpophilus mutilatus* colouration after treatment in the ORVS. The survival rate increased from 61.59% in the F1 to 73.05% in the F2 generation reared in the RR. However, a sharp decline was observed from 27.05% in F1 to 1.5% in F2 in the ORVS. There was significant difference in number of individuals between RR and ORVS in F1 and F2 (F 12.76 p= 0.001 < 0.05). The life span of F1 and F2 in the RR took about 46 days to complete; 7-21 days from adult to larvae stage, 5-15 days from the larval to pupal stage and 3-10 days from adult to pupal stage. Whereas in ORVS, F1 and F2 took about 30 and 22 days, respectively to complete their life cycles; that is 7-14, 7-14 days (adult to larval stage), 5-10, 0-5 days (larval to pupal stage) and 3-6, 0-3 days (pupal to adult stage), respectively. These data can be used to describe the changes in *C. mutilatus* due to global warming effects, as CO₂ could be one of the main factors affecting the growth and development.

Keywords: morphology, biology, climate change, insects, global warming

Introduction

The life cycle and life span of insects depend on a variety of factors including biotic and abiotic factors. Biological information regarding the life cycle, life span and mortality rates of important groups of insects, especially pests and natural enemies, are crucially important for the biological control program (Gurr et al., 2000; Khaliq et al., 2014). These parameters are usually studied under laboratory conditions where the insects are reared in cages closely representing their native habitat, with the advantage that the studied parameters and test animals are well controlled and the trials can be carried out more easily than in the field (Leppä, 2009). Insect rearing in the laboratory is important as the basis for developing a cost-effective large-scale mass-rearing in Integrated Pest Management (IPM) (Echegaray and Cloyd, 2013). Nitidulid beetles in the genus *Carpophilus* are important pests of dried fruit worldwide but have recently also become serious pests of ripening stone fruit in southern Australia (James and Vogele, 2000), as well as palms, including dates and oil palm fruits (Blumberg, 2008; Nor Atikah et al., 2019). Adult beetles damage fruit either by chewing through the skin, usually at the stem end, or entering from the sites of mechanical damage.

Taxonomists also implemented a rearing method as the first step for species identification, especially at the early stage. The insect larvae and pupae need to be reared into adult stage in order to identify the species correctly (Gibbs et al., 2015). However, insect rearing methods often require optimum conditions that correlate with their natural

habitats so that the insects are healthy, free from disease and can reproduce for future generations. Therefore, factors such as environmental conditions, food sources and rearing chambers should be appropriate, simple and cost-effective (Cohen, 2018). The rearing of nitidulid beetles in the laboratory had been studied to determine their diets (Dowd, 1987; Peng and Williams, 1990; Dowd and Weber, 1991) and under different temperature conditions (De Guzman and Frake, 2007; Cuthbertson et al., 2008) to measure their survival, development and reproduction rates (Tsukada et al., 2005; Okada and Miyatake, 2007; Meikle and Patt, 2011).

Greenhouse gases such as carbon dioxide, ethane and nitrous oxide are often associated with global warming (Cox et al., 2000; Root et al., 2003; Ainsworth and Long, 2005; Meinshausen et al., 2009). CO₂ accounts for about 82% of greenhouse gas emissions compared to other gases (EPA's Greenhouse Gas Inventory 2017). The CO₂ gas that is trapped in the atmosphere captures heat and prevents it from being released, which can lead to global warming. This phenomenon affects biological changes in many organisms such as animals and plants (Hunter, 2001; Peñuelas et al., 2002; Mondor and Tremblay, 2010; DeLucia et al., 2012; Khaliq et al., 2014). Insects are expected to be more vulnerable and sensitive to environmental changes as they are ectothermic organisms and have a short lifespan (Bale et al., 2002). For example, long-term studies on Lepidoptera found that the life cycles of this insect group are shorter as a result of global temperature changes (Roy and Sparks, 2000; Peñuelas et al., 2002; Wallis de Vries and van Swaay, 2006). The distribution of the lepidopteran species from the family Geometridae was elevated by 67 meters of altitude over 42 years due to increasing annual temperatures (Chen et al., 2009).

Previous studies, mostly conducted between 1980-90, included reports on the interactions between herbivorous insects and plants exposed to high concentrations of CO₂ gas (Agrell et al., 2000; Chen et al., 2005; Dáder et al., 2016). The oviposition behaviour of *Cactoblastis cactorum* (Stange, 1997, 1999), the feeding behaviour of the larvae of *Diabrotica virgifera* and *Helicoverpa armigera* (Rasch and Rembold, 1994; Bernklau and Bjostad, 1998) and the host-searching behaviour of mosquitoes (Gillies, 1980; Eiras and Jepson, 1991), all were reportedly influenced by the concentration of CO₂ gas as one of the most important environmental parameters. Most of these studies have been conducted in specialized rooms, especially in greenhouses or closed rooms with specific controlled parameters (Kimball et al., 2002).

In most studies, the CO₂ concentration was usually raised to twice the ambient level, or monitored between 700-720 ppm (Hughes and Bazzaz, 2001; Veteli et al., 2002; Johns et al., 2003). Therefore, our objectives were to investigate the effect of increasing CO₂ levels on the colour changes, mortality rates, life cycle and life span of the oil palm pest, the nitidulid beetle *Carpophilus mutilatus* reared under two different conditions, i.e. within the Rearing Room (RR) and in the Open Roof Ventilation Greenhouse System (ORVS). The results are expected to be very useful in predicting the future changes in the population of nitidulid beetles, particularly the pest species of our oil palm crop (Blumberg, 2008; Nor Atikah et al., 2019).

Materials and Methods

Trap design

The nitidulid beetle trap in the field was designed using a 1.0 L transparent plastic container with a cover. A 10.2 cm x 5.1 cm window was cut 5 cm from the top edge of the trap and then covered with a muslin cloth for air ventilation. The trap was filled with

dried soil up to 10 cm from its base. A rope was fastened to both sides of the trap to hang it onto a palm tree. Ripe bananas were used as food bait (*Figure 1*).



Figure 1. Trap of *C. mutilatus*

Cultural sampling of *C. mutilatus*

The sampling of *C. mutilatus* was conducted in September 2015 at an oil palm plantation in Felda Lui Muda, Negeri Sembilan, in the west coast of Peninsular Malaysia (GPS: latitude 3.013396 longitude 102.379504). The traps were used to obtain live samples of *C. mutilatus* for rearing process in the Rearing Room (RR). In the sampling area, only mature palm trees (aged 18 years and above) were selected for trapping. Three traps were hanged randomly 1 m above ground at 50 m apart from each other. The traps were inspected every three days and each trap was placed in a container containing ripe fruits and water. The trapped nitidulid beetles were taken to the Centre for Insect Systematics (CIS) laboratory, Universiti Kebangsaan Malaysia (UKM) for species identification. Only beetles from *C. mutilatus* species were selected for the rearing process in the rearing room.

Rearing process of *C. mutilates* in the Rearing Room (RR)

The rearing of *C. mutilatus* was conducted in the Rearing Room at the Biology Building, Faculty of Science and Technology, UKM following the method by Nur Hasyimah et al. (2018). The room temperature and humidity were controlled at 28-32 °C and 77-85%. A 19 x 14 x 12 cm transparent plastic container was used to rear the *C. mutilatus* specimen samples. The container was covered using a muslin cloth to prevent the beetles from escaping and a 3 cm depth of soil medium was placed inside each container to simulate the original natural habitat of *C. mutilatus* and to maintain moisture. In this study, the selected culture room was a closed system that was used exclusively for insect rearing and was also free from chemical contamination and other animals or insects. The room also had a good ventilation system to maintain the

temperatures at 28-32 °C and 77-85% humidity. Controlling temperature and humidity at appropriate levels is important to prevent fungus and other diseases that could affect the rearing process of the beetles (Singh, 1982).

A total of 200 test beetles were used for the experiment with 20 randomly selected individuals of *C. mutilatus* species placed in 10 different containers (replicates). In this study, only 20 adult beetles were placed in each culture container to prevent overpopulation, which could lead to a reduction in survival rates due to injury and lack of phosphorylation. The 3 cm depth of soil medium in the culture container was important for the pupal stage of the nitidulid beetles, which would also involve the process of fertilizing the soil during the developmental stages of their life cycle (Myers, 2001).

Once the larvae reach their optimum growth, they will excavate up to 2.5-7.5 cm depth in the soil to provide space for the pupal stage (Capinera, 2001). Water was sprayed every three days to maintain moisture in the container and ripe banana fruit was provided as a food source. The diet or food source provided is an important factor for optimal growth. In this study, mature palm fruit was given to the adult beetles as a food source. Palm fruit also provided a suitable medium for egg-laying and larval growth of the nitidulid beetles. The female would deposit its eggs in the mesocarp and in the early stage of development the larvae would eat and crawl within the fruit before exiting and searching for soil to continue with further pupal development (Glazer et al., 2007). Adult beetles would enter the palm fruit on the calcareous side and obtain food through the fibers (Blumberg, 2008).

The temperature and humidity parameters were monitored every three days using a Digital Hygrometer, i.e. a MEXTECH TM-2 model (Global Instruments, new Delhi, India) while CO₂ concentration was monitored using a CO₂ Meter, i.e. a 8802-EN-00 version (BENETECH, China). The CO₂ concentration in the RR was between 300-410 ppm.

Rearing process of C. mutilatus in the Open Roof Ventilation Greenhouse (ORVS)

The same rearing process as above was used for *C. mutilatus* trial specimens placed in ORVS following the method by Nur Hasyimah et al. (2018). The temperature and humidity of the ORVS were controlled using a computerized system and ranged between 25-45 °C and 37-87%, respectively. Abiotic parameters such as temperature, air humidity and CO₂ concentration were monitored every three days through readings on the system's screen display. In ORVS, the CO₂ treatment was given daily from 9-11 am. Pure CO₂ spraying was continued for two hours at a concentration of 800-950 ppm. After two hours, the CO₂ levels inside the ORVS was almost equal to the CO₂ levels outside. The gas was supplied through a cylinder connected to the air-conditioning system for open-air chambers and vents. The CO₂ concentration was regulated by dilution with air produced by an air blower. CO₂ analysers were used to monitor CO₂ concentrations and the automatically controlled ORVS systems monitored by the Climate Change Institute (IPI), UKM.

Monitoring the life cycle and life span of C. mutilatus

The life cycle of *C. mutilatus* was monitored daily and the emergence of the first instar larvae was recorded. The larvae were promptly isolated into different containers containing the soil medium as described earlier. Larvae that had turned into pupae were removed from the soil and placed in different containers until they became adult beetles. Observations were conducted throughout the life cycle of the beetle up to the adult stage. The number of individuals at each stage (survival rates), lifespan and body colour changes

were observed and judiciously recorded. In each stage, five individuals were selected and the colour changes were observed every two days under a microscope.

Data analysis

Two-ANOVA was used to determine any significant differences and variations between the test individuals observed between RR and in ORVS and in the F1 and F2 generations. The analysis was implemented by using Minitab17.

Results

Effect of the different CO₂ concentrations on the external morphology (body colour) of C. mutilatus in RR and ORVS

Life cycle was observed starting from the instar larval stage 1. No obvious change in body colour was observed in *C. mutilatus* reared in RR and in ORVS, even at elevated CO₂ levels. The short life cycle and morphology of *C. mutilatus* from the larval stage are as follows;

The larvae were milky-white colour on the first day of hatching and would turn into yellow when they reach the active stage and started searching for food (*Figures 2a-b*), while the pupae were white in the early stage, and would turn into brown upon reaching the final stage, and before emerging as adults (*Figures 2c-d*). Adult beetles of this species were dark brown or deep brown in colour (*Figures 2e-f*).

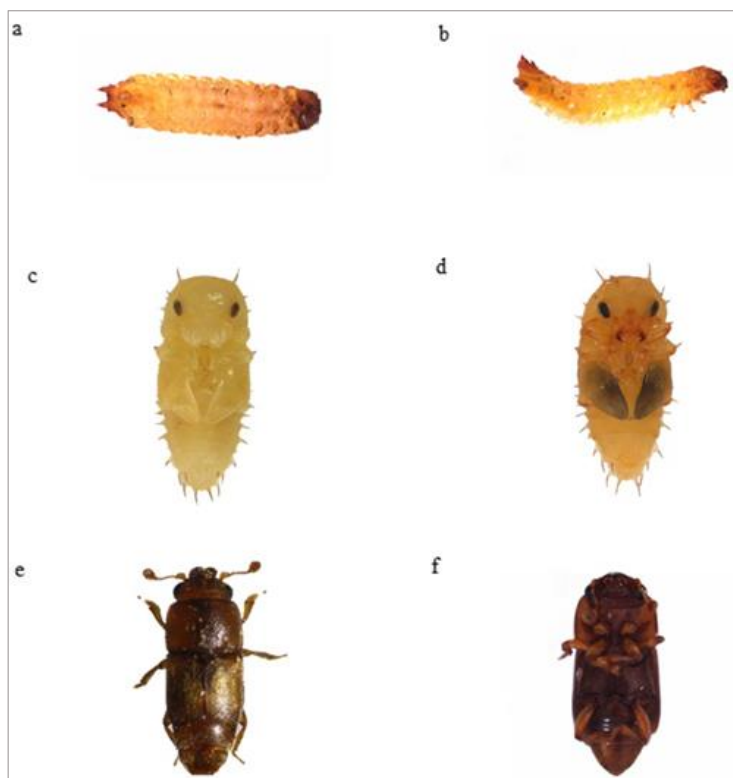


Figure 2. External morphology of *C. mutilatus* a) Larval stage (ventral) b) Larval stage (c) Early stage d) Pupa stage e) Adult stage (dorsal) f) Adult stage (ventral), taken from the ORVS system

Effect of different CO₂ concentrations on the number of individuals, life cycle and life span of *C. mutilatus*

The number of live *C. mutilatus* individuals at each developmental stage was recorded in the RR and ORVS systems. The CO₂ levels were between 300-410 ppm in the RR, and 800-950 ppm in the ORVS. In the RR, the average live individuals obtained for F1 was 2283 at the larval stage, 1727 at the pupal stage, and 1406 individuals that had successfully emerged into adults. For the F2 generation, from 1406 F1 adults, 5162 larvae, 4077 pupae and 3771 adults had successfully emerged (*Table 1*). In the ORVS, the average live individuals obtained for F1 generation was 244 larvae, 99 pupae and 66 adults. As for F2, 66 adults from F1 had produced 34 larvae and 13 pupae, while only one pupa had successfully emerged into the adult stage. In the RR, the species survival rate in the F1 generation was 61.59% and had increased to 73.05% in the F2 generation. The percentage of species survival rate in ORVS showed a sharp decline from 27.05% in F1 to 1.5% in the F2 generation. There was a significant difference in the number of individuals between RR and ORVS in the F1 and F2 generations ($F_{12.76}$, $p=0.001$, $p < 0.05$) (*Figure 3*).

Table 1. Number of *C. mutilatus* individuals of F1 and F2 in the RR and ORVS systems

Generation		1st Generation (F1)			2nd Generation (F2)		
Room	CO ₂ levels (ppm)	Larvae	Pupae	Adult	Larvae	Pupae	Adult
RR	300-410 ppm	2283	1727	1406	5162	4077	3771
ORVS	800- 950 ppm	244	99	66	34	13	1

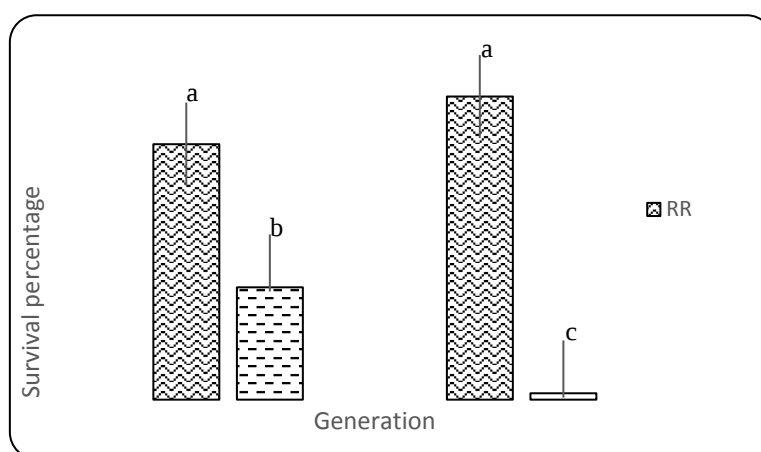


Figure 3. Percentage of *C. mutilatus* F1 and F2 survival rates in RR and ORVS systems

The life span of *C. mutilatus* F1 and F2 in RR took about 46 days to complete. In the RR, *C. mutilatus* took 7-21 days from adult to larval stage, 5-15 days from larval to pupal stage, and 3-10 days from pupal to adult stage. However, in ORVS, the F1 took about 30 days to complete its life cycle, i.e. 7-14 days from adult to larva, 5-10 days from larval to pupal stage, and 3-6 days from pupal to adult stage. However, in the F2 generation, *C. mutilatus* in ORVS had a shorter life cycle of 22 days, i.e. 7-14 days from adult to larval stage, 0-5 days from larval to pupal stage, and 0-3 days from pupal to adult stage (*Figure 4*).

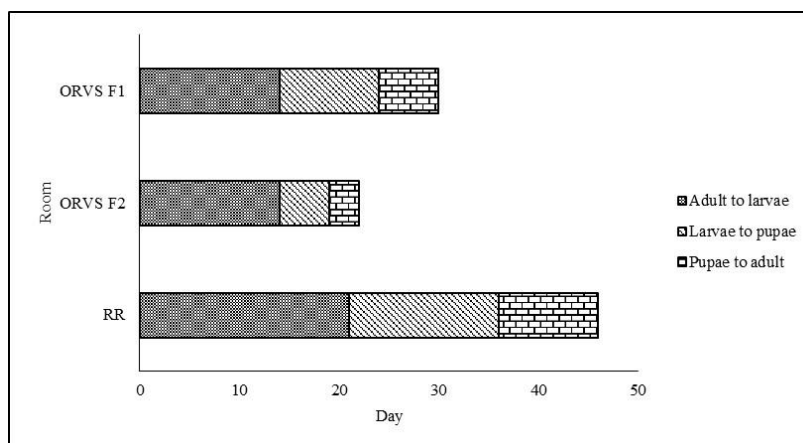


Figure 4. The lifespan of *C. mutilatus* in RR and ORVS according the developmental stages

According to Table 2, the number of individuals at each life stage in RR and ORVS also significantly differed. In RR, the adult to larva stage showed the highest number of individuals (1549 of F1 and 3147 of F2) compared to ORVS, recorded on day 8-14. In ORVS, the highest number of individuals recorded was 187 of F1 and 30 of F2, on days 0-7. The larval stage in RR was also recorded on days 6-10, with 994 and 2715 individuals of F1 and F2, respectively, while in ORVS the highest number of individuals was recorded on day 0-5, with 86 of F1 and 13 of F2, respectively. However, the pupal to adult stages in both RR and ORVS systems recorded the highest number of individuals on day 0–3, even though only one individual was recorded in F2 of ORVS.

Table 2. Number of individuals according to the developmental stages of F1 and F2 of *C. mutilatus* in RR and ORVS systems

Room	Generation	Growth development (Days)								
		Adults to larvae			Larvae to pupae			Pupae to adults		
		0-7	8-14	15-21	0-5	6-10	11-15	0-3	4-6	7-10
RR	F1	592	1549	142	556	994	117	992	305	109
	F2	1536	3147	479	1217	2715	145	2774	822	175
ORVS	F1	187	57	0	86	13	0	61	5	0
	F2	30	4	0	13	0	0	1	0	0

Discussion

According to Hunter (2001), the Free-Air Carbon Dioxide Enrichment (FACE) and Open-Top Chamber (OTC), i.e. resembling ORVS, are two effective systems for studying long term interactions of plants and insects. In both systems, the CO₂ has been raised abnormally for the purpose of the ecological study, which is believed to have significant influence on the distribution, abundance and performance of insects that feed on plants (herbivorous insects). Furthermore, the elevated levels of CO₂ in the atmosphere could exert various effects on many insects either directly or indirectly, leading to marked changes in their life cycle, physiological and behavioural aspects (Lake and Wade, 2009; Yuan et al., 2009).

From this study, no colour change was detected after exposure of the test beetle individuals to high concentrations of CO₂ in the ORVS system. According to Zeuss et al. (2014), colour changes in butterflies would involve a change from lighter to darker coloured in cooler climate, and vice versa at warmer climates when exposed to higher CO₂ levels. In our study, despite the different CO₂ levels in the culture chambers, no changes were observed based on the morphology (body colour) of each stage of the life cycle. According to Roulin (2014), the increment of CO₂ led to a rise in temperature together with the UV radiation. However, the *C. mutilatus* has been categorised as a dark beetle species which is not affected by changes in CO₂ levels due to its resistance by the production of melanin. This pigment has become a useful indicator to study the adaptation of insects to climate change. The life cycle of the *C. mutilatus* species occurs mainly inside the ripe oil palm fruit to complete the larval stages before hibernating in the soil during the pupal stage (Nor Atikah et al., 2019).

Furthermore, previous study results indicated that increase in CO₂ could also reduce insect abundance by 22%, increase the nutritional rate and life span of certain insects by 17% and 4%, as well as lowering the growth rate and weight of pupae by 9% and 5% (Stiling and Cornelissen, 2007). In our study, significant differences in the number of individuals or abundance in the RR and ORVS systems indicate that high CO₂ concentrations could have induced changes in the hatching rate of the nitidulid beetle, *C. mutilatus*. This is proven by the differences in the survival rates of this species in both culture systems.

The survival rate of *C. mutilatus* in ORVS was lower than in RR, where the rate had also decreased in the F1 and F2 generations. However, the results of this study differ from studies on *C. dimidiatus* by Odeyemi et al. (2004) and *C. hemipterus* by Gbaye and Odeyemi (2005). Dáder et al. (2016) reported that the aphid, *Myzus persicae* showed lower survival rates from eggs to adulthood and shorter larval stages in ambient conditions, which was contradictory to our own findings. The egg stage of *C. dimidiatus* showed the highest level of tolerance to high levels of CO₂ compared to the other stages in its life cycle. The mortality rate of the eggs was 13.3% lower than that of the larvae and pupae at 50.0% and 33.3%, respectively, while 100% of the adult beetles died after exposure to high CO₂ levels for six hours (Odeyemi et al., 2004).

Carpophilus hemipterus also recorded the lowest egg mortality rate of 26.7% compared to 86.7%, 100.0% and 90.0% in larval, pupal and adult stages, respectively. However, in our study the egg stage was not successfully obtained from the emerged adults. Most insect eggs require less oxygen to survive compared to the larval, pupal and adult stages. The egg tolerance rate is due to an impermeable layer of egg coral structure that is not present at other stages (Chapman, 1971). In this study, the eggs stages were unsuccessfully collected and thus, not evaluated. However, the percentage of emergence in the pupal and adult stages were higher in larval, continued with pupal and then in adult stages in both conditions and in both generations.

Insects adapt differently when their habitat is changed. At higher CO₂ concentrations in ORVS, the life span of *C. mutilatus* was shorter than in the RR. The *C. mutilatus* F1 and F2 generations also survived, the second generation having a shorter life span than the first generation. The effect of increasing CO₂ concentration either in ambient conditions or in the system is usually associated with the interactions of plants and insects. This is because by increasing CO₂ the nitrogen cycle is affected and thus, resulting in a decrease in the C:N ratio in the plants (Ainsworth and Long, 2005; Oehme et al., 2013; Ryan et al., 2014). As a result of the changes, macronutrients such as calcium, magnesium

and phosphorus would decrease due to the lack of water supply from the soil (Taub and Wang, 2008). Therefore, the lack of nutrients and plant quality due to the changes in CO₂ concentration would indirectly affect insects through their nutritional responses (Hughes and Bazzaz, 2001; Himanen et al., 2008; Stiling et al., 2013).

Our results showed that the life span of *C. mutilatus* was shorter in ORVS compared to the RR, indicating that the life span of the beetles treated with elevated CO₂ above the ambient levels had been shortened, and this effect was not limited to F1 but was also observed in the F2 generation. However, this result was contradictory to those of previous studies, which reported that insects tended to reduce their growth rates and extend their life span in order to adapt to high CO₂ levels (Goverde and Erhardt, 2003). Insects from *Helicoverpa armigera* and *Orygia leucostigma* species also reportedly extended their lifespan when exposed to elevated CO₂ levels (Agrell et al., 2000; Chen et al., 2005).

The rise of CO₂ levels as a greenhouse gas (GHG) would affect the atmospheric temperature, and both abiotic factors could significantly affect the growth and development of many insect species (Agrell et al., 2000; Goverde and Erhardt, 2003; Mondor and Tremblay, 2010). Although some species are not affected by CO₂ changes, but as exothermic organisms, most insects are more sensitive and respond quickly to changes in temperature that affect their life cycles and growth rates (Bale et al., 2002). Insects also respond to rising temperatures by increasing their rates of growth, reproduction and mortality (DeLucia et al., 2012).

The development of nitidulid beetle species, namely *C. humeralis*, *C. hemipterus* and *C. mutilatus* at different temperatures had been studied by James and Vogele (2000), who reported that the lifespan of these three species were shorter at higher temperatures, i.e. 14-18 days at 32.5 °C compared to 47-65 days at 20 °C. These findings, however, contradictory to our results that in RR under ambient conditions (28-32 °C), the total lifespan of *C. mutilatus* was 46 days, whereas for ORVS it was 30 days. The mortality rate for each species was the lowest at temperature between 25-30 °C. The life span of *C. hemipterus* also exhibited similar effects when exposed to different temperature ranges where the growth rates for eggs, larvae and pupae were longer at low temperatures (18 °C) than at high temperatures, (30 °C). However, the mortality rate has increased with increasing temperature (Tsukada et al., 2008). At low temperatures of -10 °C to -8 °C, the mortality rates of *C. mutilatus* and *C. hemipterus* were higher with 100% mortality (Donahaye et al., 1991).

Conclusion

The nitidulid beetle *C. mutilatus*, was selected as a model species to evaluate the colour changes, survival rate and life span of *C. mutilatus* (Coleoptera: Nitidulidae) in different concentrations of carbon dioxide (CO₂). This species is interesting because its life cycle from the egg to larval stages is spent inside the oil palm fruit. From this study, no significant changes were observed in the coloration of the larval to adult stages of *C. mutilatus* after exposure to treatment with high ambient CO₂ levels. In the RR, the species survival rate in the F1 generation was 61.59% and had increased up to 73.05% in the F2 generation. However, the survival rate in ORVS showed a sharp decline from 27.05% in F1 to 1.5% in F2 generation. Two-ways ANOVA shows there was significant difference in number of individuals between RR and ORVS in F1 and F2 (F 12.76, p=0.001, p < 0.05). The life span of F1 and F2 of *C. mutilatus* in RR took about 46 days to complete; i.e. 7-21 days from adult to larval stage, 5-15 days from larval to pupal stage, and 3-10

days from adult to pupal stage. However, in ORVS, the F1 and F2 generations of *C. mutilatus* took about 30 and 22 days, respectively, to complete their life cycles; i.e. 7-14, 7-14 days from adult to larval stage, 5-10, 0-5 days from larval to pupal stage, and 3-6, 0-3 days from pupal to adult stage, respectively. This data can be used to describe the changes in *C. mutilatus* caused by global warming effects, as CO₂ could be one of the main factors affecting the species development. Further ecological study in the field (oil palm plantation) is suggested to relate the growth and development of the nitidulid beetles with other abiotic factors such as temperature, humidity, light intensity and also the most important parameter, i.e. CO₂ concentration in order to validate the effects on and biological responses of *C. mutilatus* to climate changes in the natural environment.

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