

CO₂ EFFECTS ON LARVAL DEVELOPMENT AND GENETICS OF MEALWORM BEETLE, *TENEBRIO MOLITOR* L. (COLEOPTERA: TENEBRIONIDAE) IN TWO DIFFERENT CO₂ SYSTEMS

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Abstract. *Tenebrio molitor* has never been used as a model species for studying global warming effects. The objectives of this study were to measure the larval development and genetics of *T. molitor* under a free air CO₂ enrichment (FACE) system, open roof ventilation greenhouse system (ORVS) and a rearing room system (RR). The correlation coefficient analysis showed that the head width: body length was the best character to measure the development (0.465, 0.940 and 0.893) in all systems. Our results show that there were no significant changes in the larvae samples under RR condition, however, slight and moderate changes were observed under FACE and ORVS. Neighbour-joining analysis using cytochrome c oxidase subunit I sequence revealed the genetic data parallel with the results of the correlation coefficient. The FACE F1 progeny showed the slowest development (0.080±0.018 mm) during 0-14 days of larval development, while the most rapid before pupation occurred in the ORVS F1 (0.1205±0.0028 mm). No significant differences were noted between the systems for 0-14 days and before pupation, except for RR F1 vs ORVS F1 and ORVS F1 vs FACE F1 ($p = 0.000$, $p = 0.002$). These data can be used to clarify the changes in *T. molitor* due to global warming effects, as CO₂ could be one of the factors affecting the larval development.

Keywords: correlation, Tenebrionidae, global warming, FACE, development growth, DNA, neighbour-joining

Introduction

Tenebrio molitor L. (Coleoptera: Tenebrionidae), also known as yellow mealworm, has a high reproductive capacity and the capability to sense and avoid toxic fungi-colonised diets (Morales-Ramos et al., 2011). This insect is also viewed as a major insect pest because it attacks several classes of stored grains, milled cereals, meat scraps, dead insects, flour and is even found feeding on sparrow droppings (Ye et al., 2001). Traditionally, *T. molitor* larvae have also been used as a food source for pets, including reptiles, fish, and birds, because the larvae are a rich source of protein, vitamins, essential amino acids, minerals and essential fatty acids (Tang et al., 2012).

The increment of CO₂ concentration affects the performance of insect behavior, life history, and population abundance. Williams et al. (2003) showed that long-term exposure for an increment of 3.5 °C on *Lymantria dispar* shortened the insect development by reached pupation earlier. Consumption rates for *Octotoma scabripennis*

were higher than *O. Championi* when given CO₂-grown foliage under lower temperature (Johns et al., 2003). Research done by Chen et al. (2005) revealed that fecundity of *Aphis gossypii* was increased through successive generation with increasing of CO₂ concentration.

Based on the review by Epenhuijsen et al. (2002), mortality rate shown by *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) increased rapidly as the concentration of CO₂ increased from 60 to 90% at elevated temperatures between 38-42 °C. Furthermore, the developmental delay of cowpea bruchids was more severe under 2% O₂ + 18% CO₂ compared to 10% O₂ + 10% CO₂ (Cheng et al., 2012). Insect herbivore which consumed plant lowered nutritional quality as food will cause a reduction in their performance, including by reducing their growth rates and prolonging their development time (Goverde and Erhardt, 2003). Indirectly, the mortality imposed by natural enemies increase (Stiling et al., 2003), finally reducing the abundance, diversity, and richness of herbivore if compared to ambient CO₂ environments (Cornelissen, 2011).

Evaluation and monitoring of insects morphological changes can be carried out through the morphometric analysis. For example, the measurement process on the capsule head size was conducted after the discovery of the sub-fossil weevil in that area. As a result, based on measurements and analysis performed, the sub-fossil remains belong to the *Ectemnorhinus* group of weevils. A total of 15 characters of weevil species have been measured and used as references for further systematic and population studies including total body length (TL), pronotum breadth (PB), femur length (FL), etc. (Janse van Rensburg, 2006). Based on the morphological and morphometric studies on insect herbivores by Stiling and Cornelissen (2007), it shows that their development time increase about 13.87%, while their relative growth rate and pupal weight are significantly decreased about 8.3% and 5.03% after being exposed to the elevated atmospheric CO₂.

One of the statistical methods used to show the relationship and the association between two variables is the analysis of Pearson's correlation coefficient or simply referred as the correlation coefficient (Camargo et al., 2011). A study done by Loudon (1989) shows that a weak positive correlation was detected between tracheal sizes within individual larvae after correcting for larval size, which treated with a different level of oxygen separately ($r = 0.53$). Other study conducted on homopterous insects also indicates that relationship between wingbeat frequencies with wing loading to be significantly correlated only in insects weighing more than 0.03 g. The lack of correlation between these two parameters in small insects with less than 0.03 g maybe due to different flying strategies (Byrne et al., 1988). So, in the present study, the similar analysis can be used to identify whether different CO₂ level affects the characters of *T. molitor* larvae.

According to Brown et al. (2003), *Tribolium castaneum* is destined to become one of the three most important insect model systems as its genetics has convenient genetic setup. Several studies were conducted to create evolutionary trends from the family Tenebrionidae, but no phylogenetically informative molecular data are available for this genus (Mestrovic et al., 2006). Sequence analysis has been performed for a 642 bp fragment of the most conserved part of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene in eight species of *Tribolium*, as well as in two outgroup species, *Pimelia elevata* and *T. molitor* (Mestrovic et al., 2006).

Some studies were done on other insects as subjects or model species predict similar results in *T. molitor*. Therefore, the aims of the present study were to (1) determine the correlation and differences between morphological characters of *T. molitor* larvae, due to changes in CO₂ concentration in three different systems, and (2) determine the genetic changes in *T. molitor* larvae from FACE, ORVS and RR (as a control) populations.

Materials and methods

The study was conducted at two enriched-CO₂ systems, including; FACE (N 02°55.331', E 101°46.965') and ORVS (N 02°55.145', E 101°46.465'), which was located at Universiti Kebangsaan Malaysia (UKM).

Rearing room system (RR)

The RR system was set up as a control room for observation of *T. molitor* during this experiment. The rearing room is a closed room which is free from insecticide and chemical contamination and is designed for rearing purposes. This room is specifically used to deter other animals from approaching the sample and to reduce any large impacts due to external temperatures and humidity. Larval and adult mortality might be caused by the temperatures higher than room temperature (Singh, 1982). The room size is 214 × 166 inch and samples were placed on the bench size 134 × 36 × 34 inch. The CO₂ concentration in this system can reach about 441-553 ppm.

Free air CO₂ enrichment system (FACE)

The FACE system is a new system which provides an environment with a high CO₂ level. It enables the design of a model to show how ecosystem respond to proliferating CO₂ in the Earth's atmosphere (Norby and Zak, 2011). The components of the FACE system include a smart control panel, a control system (valve 1, valve 2, valve 3 and buzzer), sensors (temperature, humidity, carbon dioxide and wind speed), a server, internet connection, PC application and mobile application. This system is operated by support from Xbee wireless sensors on four EZ sensor nodes and one wind speed (WS) sensor node. All four EZ sensor nodes contain built-in temperature, humidity, and carbon dioxide sensors, while the WS sensor node has wind speed meter. Sensor data were sent to the server immediately after receipt by Sensor Control Programmable (SCP) using gsm/gprs communication. Android and desktop applications were used to view the data stored on a cloud server database.

Carbon dioxide emissions were controlled by opening and closing a pipe valve at 1 h intervals when the wind speed is below 15 km/h. Four carbon dioxide tanks were used to supply carbon dioxide and were changed for every 43 days. Valve 1 was opened for 5 min at the Real Time Clock (RTC) hour. Later, valve 2 was opened for 5 min after 10 min at the RTC hour and valve 3 was opened after 20 min at the RTC hour for 5 min. During the process of carbon dioxide gas emission, the buzzer was on, the date and time on the LCD were adjusted using the menu and keypad, and sensor data were sent to the server, together with a timestamp. The original CO₂ concentration that has been released into the FACE system in the range of 800-950 ppm. FACE system of UKM shows in *Figure 1b*.

Open roof ventilation greenhouse system (ORVS)

The ORVS system (Fig. 1a), had an average temperature of 34 °C and 63% humidity. The light intensity was 94-95% higher than the ambient level. CO₂ was provided by continuous spraying of pure carbon dioxide automatically from 9 am to 11 am for 2 h every day and was stopped once the concentration of CO₂ reached about 800-950 ppm. It was provided via a CO₂ cylinder connected to the air delivery system of the open roof ventilation and air blower. The CO₂ concentration in the chamber was monitored and managed with a CO₂ analyzer. After 2 h, the CO₂ concentration in the chamber and in ambient air was almost the same. The ORVS was automatically controlled by a system set up by the Climate Change Institute (IPI), UKM.



Figure 1. Elevated CO₂ systems (a) open roof ventilation greenhouse system; and (b) free air CO₂ enrichment system

Figure 2 shows the arrangement of the samples in ORVS and FACE during the process of rearing and monitoring.



Figure 2. Experimental design of *T. molitor* study in (a) open roof ventilation greenhouse system, and (b) free air CO₂ enrichment system

Sample identification and preparation

Samples of *T. molitor* larvae were obtained from a local supplier from Bandar Baru Bangi, Selangor, Malaysia. The species identification was confirmed morphologically using the species key by Bousquet (1990).

Rearing process of Tenebrio molitor

Tenebrio molitor larvae were reared in the Cytogenetic 2 Laboratory, Universiti Kebangsaan Malaysia (UKM), from October 2015 until November 2015. The larvae samples were placed in 40 × 28 × 24 cm plastic aquariums and fed with oats and cucumbers as a food source during the rearing process (Siemianowska et al., 2013). Some parameters, including temperature, humidity, and CO₂ concentration were recorded. The samples were observed and monitored until the emergence of the *T. molitor* adults.

Isolation of samples of Tenebrio molitor adults

The emerged *T. molitor* adults were collected from the rearing process and were separated into 30 of 19 × 14 × 12 cm aquariums. Forty randomly picked individuals were put into each aquarium, together with a 4 cm height of sawdust at the bottom and a 4 cm height of sifted soil. Ten aquariums were placed in each of the three systems (Free Air CO₂ Enrichment System (FACE), Open Roof Ventilation System (ORVS) and Rearing Room System (RR)). Larvae were collected two weeks later (14 days) and before pupation.

CO₂ treatment at different systems

The data for CO₂ from each system, as well as other parameters, including valve number, temperature, and humidity, were recorded at every sampling time (every 2 weeks).

Monitoring of Tenebrio molitor adult (parent) to larval stage (F1)

Samples of adult *T. molitor* were observed once a week until the first group of larvae emerged. The survival of each individual from each aquarium was monitored and recorded (Crawley, 2007). The mortality rate of adults was also recorded by collecting the dead *T. molitor* individuals during the observation.

Sampling of Tenebrio molitor larvae and adults

Aquariums containing *T. molitor* samples exposed to high CO₂ concentration were randomly collected every week. A forcep was used to collect the dead adult bodies and live larvae, which were placed into small containers and sorted according to the aquarium. The randomly picked larval samples consisted of about 40-60 individuals per session, depending on the larval size. Dead *T. molitor* adults were brought to the laboratory and recorded.

Morphometric measurement of Tenebrio molitor

Fifty larvae were picked randomly from each system and were used for morphometric measurements. The images of larvae and adults were captured and measured with a microscope (Dinolite 1.2). The characters of the larvae including the head width (HW), head length (HL), body width (BW) and body length (BL) were measured in millimeter. Generally, the width of the head capsule was measured because it exhibits distinct variations between larval stages (Hsia and Kao, 1987).

DNA isolation, PCR amplification, DNA purification sequencing analyses

DNA was extracted from larvae from each treatment using a NucleoSpin® DNA Insect kit. The kit combines enzymatic lysis by utilizing mechanical disruption of cell walls with the NucleoSpin® Bead Tubes (Macherey-Nagel, 2016). The list of isolated samples, type of treatment and age of *Tenebrio molitor* larvae used for genetic study using cytochrome c oxidase subunit I (COI) and phylogenetic analyses were summarised in *Table 1*. A pair of primers designed by Folmer et al. (1994) [LCO1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3 (Forward) and HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3 (Reverse)] was used to amplify the COI by singleplex PCR using a MyGene MG96G Asthermalcycler.

The PCR conditions were as follows: 95 °C for 3 min as the pre-denaturation, followed by denaturation with 95 °C for 1 min, an annealing process of about 1 min, elongation and final elongation at 72 °C for 10 min 30 sec. The whole PCR process consisted of 35 cycles over 150 min. The PCR products were electrophoresed on a 1.5% agarose gel and the bands corresponding to the target PCR products were purified using the Geneaid Purification Kit. All PCR products were sent to the sequencing service company, First Base Sdn. Bhd., Petaling Jaya, Selangor.

Table 1. List of samples, type of treatment and age of *T. molitor* larvae used for genetic study and phylogenetic analyses

No.	Code	System	Age (day)	Accession No
1	5RRF1	RR	71-84	MF155950
2	6RRF1	RR	71-84	MF155953
3	1ORVSF1	ORVS	85-98	MF155951
4	2ORVSF1	ORVS	85-98	MF155952
5	7ORVSF1	ORVS	85-98	MF155954
6	8ORVSF1	ORVS	85-98	MF155955
7	9ORVSF1	ORVS	85-98	MF155956
8	13ORVSF1	ORVS	85-98	MF155959
9	4FACEF1	FACE	43-56	MF155949
10	11FACEF1	FACE	43-56	MF155957
11	12FACEF1	FACE	43-56	MF155958
12	14FACEF1	FACE	43-56	MF155960
13	15FACEF1	FACE	43-56	MF155961
14	16FACEF1	FACE	43-56	MF155962

RR = rearing room; FACE = free air CO₂ enrichment; ORVS = open roof ventilation greenhouse

Pairwise alignment, basic local alignment search tool analysis (BLAST) and neighbour joining analysis

All the sequence-based samples from the FACE, ORVS and RR systems were aligned using Clustal W to determine the similarity of characters between sequences (Thompson et al., 1994). The pairwise alignment results were optimized by manually editing these sequences using MEGA7. A BLAST search showed a maximum hit for the respective species only, as available in GenBank

(<http://www.ncbi.nlm.nih.gov/genbank>). The phylogenetic tree was constructed using Neighbour Joining (NJ) using PAUP 4.0 and the Kimura-2 parameter.

Analysis

Correlation and regression analysis for all parameters (characters) in all systems were performed using Excel 2013 and SPSS version 19 software. *T*-tests and standard errors were analyzed using Minitab 17 software.

Environmental parameters control

Free air CO₂ enrichment (FACE) system

FACE system is an open system that has been created to conduct research on the effects of elevated atmospheric CO₂ concentrations, especially on plants. However, the system has been built by maintaining its environmental parameters including temperature, humidity, and wind, etc., which may reduce the effects of the CO₂ (McLeod and Long, 1999). According to Ainsworth and Long (2005), FACE system is the best system to carry out studies related to the elevated atmospheric CO₂ level in plants and ecosystems. This is due to its characteristics as an open system without barrier, which provides or mimics the natural environment except for the concentration of CO₂.

Therefore, the results obtained from the experiments are very accurate and less bias. Based on previous studies, there are some data related to CO₂ concentration levels were recorded in the FACE systems, which is in the range between 475-600 ppm (Ainsworth and Long, 2005), 550-600 ppm (Long et al., 2006) and 550-580 ppm (Norby and Zak, 2011). These concentrations are at the similar rate as enrich-CO₂ recorded during these sampling activities. The CO₂ gas injection and release processes in the FACE system are conducted every 10 s by the two sets of the four tubes, where the process is strongly influenced by wind speed (m/s) (Okada et al., 2001).

Open roof ventilation greenhouse system (ORVS)

In contrast to FACE systems, an open roof ventilation greenhouse (ORVS) is a closed system with movable shade or open roof vents which are controlled by a computer system and a good PPFD sensor (Albright et al., 2000). Abiotic factors including temperature (°C), humidity (%) and CO₂ concentration (ppm) do not significantly affect the performance of the system against wind speeds (Boulard and Draoui, 1995). The monitoring process is carried out inside and outside the greenhouse on humidity and temperature, where both parameters were measured using Psychrometers. The ventilation process has to be carried out by the greenhouse during a large proportion of the daytime to regulate the temperature (Boulard and Draoui, 1995) and also to ensure that the humidity can be reduced (Harmanto, 2006).

When relative humidity inside the greenhouse exceeded 85%, 25% of the overall roof size was opened to lower the humidity rate (Sanchez-Guerrero et al., 2005). The gas tracer was supplied during closed vent with a concentration greater than 200 ppm. To ensure that the gas is mixed in the air uniformly, the vent is left closed for a while after the gas is released in the greenhouse (Kittas et al., 2005). In order to maintain the level of CO₂ inside and outside the greenhouse during the process of ventilation, the CO₂ was injected continuously and maintained at a concentration about 700-

800 mmol⁻¹, where it usually conducted when the vent roof of the greenhouse is closed in the early morning and the late afternoon (Nederhoff, 1994).

Based on the information above, FACE system and ORVS are able to maintain and control other parameters such as temperature and humidity, and provide ambient conditions except for elevated CO₂ during the experiment. It is able to directly observe the effect of CO₂ concentration towards *T. molitor* larvae caused by different CO₂ concentration without affected by other environmental parameters in both systems.

Results

Correlation coefficient and significant studies on control and treated Tenebrio molitor larvae

Correlation coefficient analysis shows the association between characters of *T. molitor* larvae from all systems (Table 2).

Table 2. Correlation coefficients among *Tenebrio molitor* larvae (F1) characters in FACE (i), RR (ii) and ORVS (iii)

Characters/System	FACE	RR	ORVS
HW vs HL	-0.018	0.961**	-0.21
HW vs BL	0.465**	0.940**	0.893**
HW vs BW	0.239**	0.956**	0.795**
HL vs BL	0.001	0.924**	0.001**
HL vs BW	-0.02	0.929**	-0.23
BL vs BW	0.328**	0.979**	0.684**

* and **: significant at the 0.05 and 0.01 level of probability, respectively

HW = head width (mm); HL = head length (mm); BW = body width (mm); BL = body length (mm); RR = rearing room system; FACE = free air CO₂ enrichment system; ORVS = open roof ventilation greenhouse system

A weak positive linear and highly significant correlation was evident between HW and BW ($r = 0.239$, $p = 0.000 < 0.01$) and BL ($r = 0.465$, $p = 0.000 < 0.01$), and between BL and BW ($r = 0.328$, $p = 0.000 < 0.01$). A correlation similarity was also found among characters in the ORVS F1 and FACE F1, where a negative correlation was found between HL and HW ($r = -0.018$, $p = 0.794 > 0.01$) and BW ($r = -0.020$, $p = 0.772 > 0.01$) (Table 2i).

The data on correlation coefficients between various parameters of RR larvae were presented in Table 2ii. All characters demonstrated strong positive linear correlations with significance at the 99% confidence level. The highest r value was between BL and BW ($r = 0.979$, $p = 0.000 < 0.01$), while the lowest r value was found between HL and BL ($r = 0.924$, $p = 0.000 < 0.01$). No negative correlation was found in any characters of the RR F1 larvae.

A strong positive linear correlation was noted between HW and BL ($r = 0.893$, $p = 0.000 < 0.01$), a moderate positive linear correlation with BW ($r = 0.795$, $p = 0.000 < 0.01$) and a moderate positive linear correlation between BL and BW ($r = 0.684$, $p = 0.000 < 0.01$). The result is accurate with a 99% confidence level for a significant correlation shown for all characters. A negative correlation was obtained

between HL and HW ($r = -0.21$, $p = 0.679 > 0.01$) and BW ($r = -0.23$, $p = 0.660 > 0.01$) (Table 2iii).

Developmental size of *Tenebrio molitor* samples from FACE, ORVS, and RR systems

The developmental size (HW/BL) of *T. molitor* larvae was measured at 0-14 days among the three systems. Figure 3 shows that FACE F1 had the lowest mean development (0.080 ± 0.018 mm), while ORVS F1, at 0.1293 ± 0.032 mm, had the highest. The descriptive error bars in a graph of experimental biology were used to determine whether the error of a reported measurement fits within the normal range. The type of error bar can be used to determine the significant differences among samples (Cumming et al., 2007). Figure 3 shows significant differences in the development of *T. molitor* larvae among systems (Table 3).

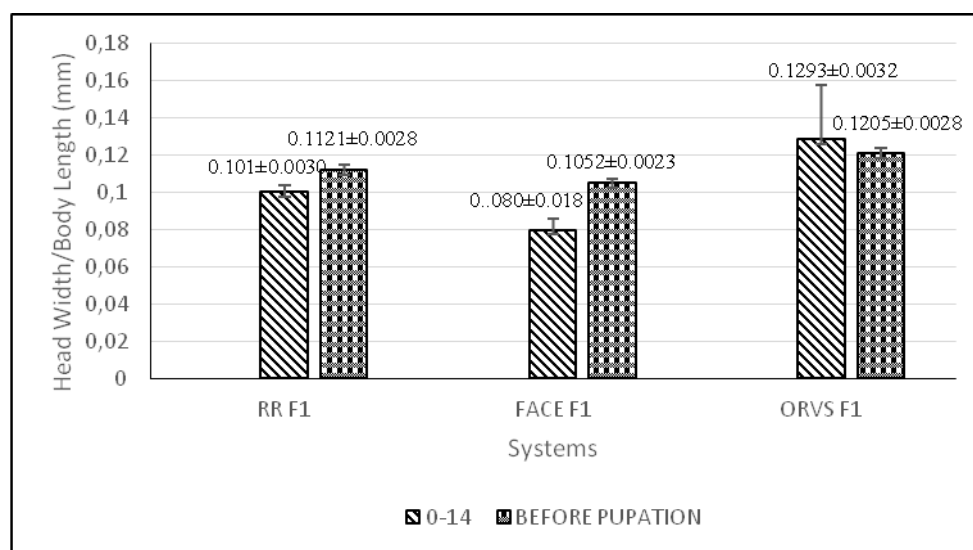


Figure 3. Developmental size of *Tenebrio molitor* larvae at 0-14 days and different days before pupation from three different systems. (RR = rearing room; FACE = free air CO₂ enrichment; ORVS = open roof ventilation greenhouse system)

The data on development size of *T. molitor* larvae before they get pupated was depicted in Figure 3. The highest mean developmental size of larvae was recorded in the ORVS F1 (0.1205 ± 0.0028 mm), followed by RR F1 (0.1121 ± 0.0028 mm) and FACE F1 (0.1052 ± 0.0023 mm). Overall, the size development of larvae before pupation at RR F1 and FACE F1 increase, while in ORVS F1 larvae showed a decrease compared to 0-14 days.

The larval development of *T. molitor* samples showed significant differences between the RR F1 vs ORVS F1 (0-14 days) only with $p = 0.000$ ($p > 0.05$). Comparison of RR F1 vs FACE F1 ($p > 0.05$, 0.078) and ORVS F1 vs FACE F1 ($p > 0.05$, 0.059) at 0-14 days revealed no significant difference based on the t-test analysis. Therefore, the t-test showed no statistically significant difference between RR F1 vs ORVS F1 ($p > 0.05$, 0.493) or RR F1 vs FACE F1 ($p > 0.05$, 0.565) before pupation, while the difference between ORVS F1 vs FACE F1 was statistically significant ($p < 0.05$, 0.002) (Table 3).

Table 3. Significant differences in *Tenebrio molitor* development (at 0-14 days and before pupation)

System/Significant difference	RR F1 vs FACE F1	RR F1 vs ORVS F1	ORVS F1 vs FACE F1
0-14 days	0.078	0.000	0.059
Before pupation	0.565	0.493	0.002

RR = rearing room; FACE = free air CO₂ enrichment system; ORVS = open roof ventilation greenhouse system

Genetic changes in *Tenebrio molitor*

Tenebrio molitor DNA samples were amplified using PCR to determine the sequence changes after treatment with CO₂. Phylogeny is a relationship classification between two species that can be represented as a phylogenetic tree (Rizzo and Rouchka, 2007). Neighbour-joining (NJ) trees were created from DNA sequencing analyzed by First Base Sdn. Bhd.

Figure 4 indicates the similarity of DNA sequences from *T. molitor* induced with high CO₂. Two clades are evident: both CLADE A and CLADE B, with 96% bootstrap values, respectively. The samples of ORVS F1 larvae induced with CO₂ were separated from the RR F1 samples (which served as a control), except for some samples of FACE F1 which found in both clades. The RR F1 was included in one subclade, with a 97% bootstrap value, with *Tenebrio obscurus* and *Tenebrio opacus* samples as outgroups. In summary, the genetic data parallel with the results of the correlation coefficient.

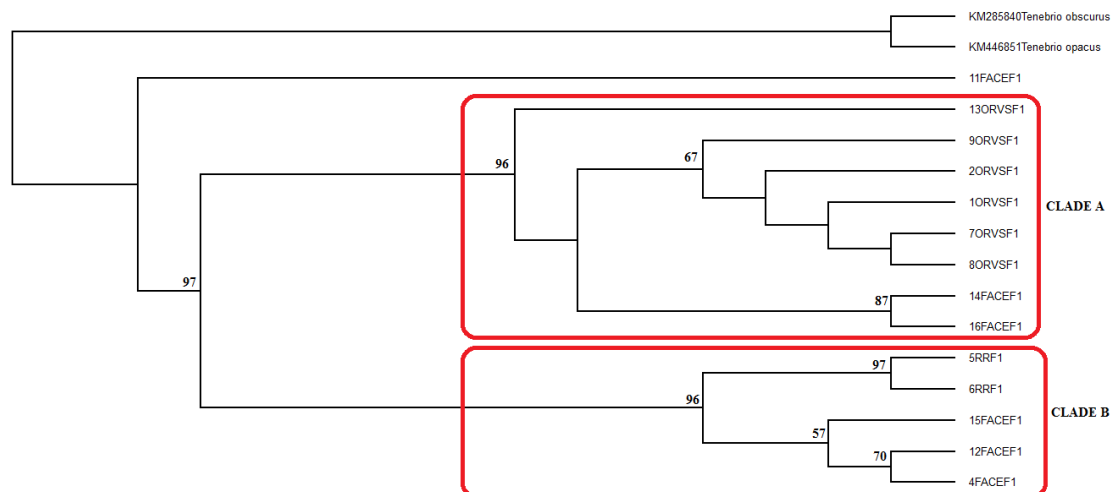


Figure 4. Neighbour joining (NJ) tree of *Tenebrio molitor* derived from NJ analyses with bootstrap values above nodes (1000 replications). (RR = rearing room; FACE = free air CO₂ enrichment system; ORVS = open roof ventilation greenhouse system)

The sequence extraction revealed 11 FACE F1 with higher nucleotide dissimilarity from the other samples when outgroups were excluded. Certain sites, including 103 (C, T), 111 (A, G), 381 (A, G), 432 (A, G), 436 (T, C), 582 (C, T), 639 (A, G) and 642 (A, G), showed a division into two types of nucleotides in almost equal quantities (Table 4).

Table 4. Sites with nucleotide changes excluding outgroup sequences

Plate no.	Nucleotide sites							
1	19	21	22	30	43	44	51	60
2	103	111	112					
3	201	237						
4	249	297	301					
5	329	330	357	381				
6	412	426	432	436	438	444	477	
7	489	495	501	519	540	558	561	
8	582	633	636	639	642	652		
9	683	685	686	687	688			

*Excluding outgroup sequences

Discussion

Larval development was measured in this experiment as a parameter to investigate the effects of exposure to elevated CO₂ on insect species, specifically in terms of prolongation of development time and changes in their growth rate (Goverde and Erhardt, 2003), longevity (Werner et al., 2006; Coll and Hughes, 2008), body size (Roth and Lindroth, 1995), adult weight (Coll and Hughes, 2008), mortality (Epenhuijsen et al., 2002), and hatch rate or pre-oviposition time (Peltonen et al., 2006; Coll and Hughes, 2008). Morphological parts were assessed as these are more conspicuous than physiological aspects, therefore more easily measured. These developmental time changes, lower survivorship, reduced adult weight, etc. are not observed only in this insect, but are seen in herbivores as well. For example, *Helicoverpa armigera*, which consumes the fruits of many crop plants, undergoes extensive mortality and delays in development time in response to high CO₂. Hence, the larvae are smaller when feeding on plants grown in elevated CO₂ than in ambient air. This response is due to the reduction in N content of the plants, which directly affects their quality, even though plant size is significantly larger when compared to plants grown at ambient CO₂ (Coll and Hughes, 2008).

The developmental results presented here were further strengthened by *t*-tests conducted on larval development between RR F1 vs ORVS F1 (0-14 days) and ORVS F1 vs FACE F1 (before pupation). Significant difference was found, with $p = 0.000$ (0-14 days) and $p = 0.002$ (before pupation). This finding indicates that increases in CO₂ above ambient levels have caused significant effects on *T. molitor* development, especially in the ORVS F1. It can be observed from the comparison of larvae development, where the development of ORVS F1 only shows a significant difference when compared to other systems.

Elevated CO₂ concentration also affects the development and growth of insects indirectly, such as through their diet. *Myzus persicae* is herbivore insect species that use pepper plants as a source of food. Based on previous research, the fitness level of *M. persicae* was decreased when feeding on the pepper plants grown under higher CO₂ concentrations. Similar results were recorded on species of Brassicaceae (Oehme et al., 2011) such as *Brevicoryne brassicae* on Brussels sprout (Ryan et al., 2014), and *Acyrtosiphon pisum* on the broad bean (Ryan et al., 2014). Other than that, elevated CO₂ also affects their morphology and physiology by reducing fertility and producing a

fewer number of offspring, as well as increasing chewing insects development duration (Dáder et al., 2016).

Different results are shown between RR F1 vs FACE F1 where there was no significant difference in 0-14 days or before pupation. It is due to the relatively long duration of exposure to high CO₂ concentrations by *T. molitor* larvae in the ORVS resulting in faster interruption of its development. Indirectly, the development time and r_m of the grain aphid, *Sitobion avenae* were not affected when feeding on winter wheat which has been exposed to high CO₂ concentration of 700 ppm (Awmack et al., 1996). According to Zalucki et al. (2002), insect larval stages are suitable to be used as a subject study because it is very sensitive to any parameter changes in the environment. Limited information on the direct responses of insects to controlled atmosphere treatments has been recorded especially on their physiological and biochemical parts (Zhou et al., 2001), developmental time and consumption of herbivores (Yin et al., 2010).

However, even though no radical CO₂ effect was observed, high CO₂ exposure still affected the various larval parameters and thus reflected in correlation results, as evident by the negative correlation of HL with HW and with BW in both ORVS F1 and FACE F1 larvae. However, the exposure of the next generations of *T. molitor* to increased CO₂ concentrations is expected to increase the possibility of effects on species development, especially changes in their morphological and molecular parts. A study was done by Yin et al. (2010) on cotton bollworm, *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) larvae for first and second successive generations when they were fed on an artificial diet or C4 plants (maize) grown under two levels of CO₂. It has resulted in prolongs larval duration, lower fecundity and reduced r_m of cotton bollworms. Furthermore, it also increased the consumption rates of the individual, decreased the total consumption of their first generation populations but increased it in the second generation.

The *T. molitor* larvae from the FACE, RR and ORVS systems showed positive correlations among several characters except for the correlation coefficient between HL vs HW, and HL vs BW, which were negatively correlated. Comparison of the correlation coefficient data, using the RR system as a control with ambient CO₂ concentration revealed a strong positive linear correlation for larval development between all characters at the 99% confidence level. Consequently, larvae exposed to higher CO₂ concentration above ambient levels in the ORVS and FACE systems showed moderate, weak and negative correlations between characters.

According to Loudon (1988), the final size of the larvae should be highly correlated with the size of pupae and the adult size in most insects. A high degree of correlation was observed for *T. molitor*, where the weight of a starved adult is highly correlated with pupal weight ($r^2 = 0.85$, $n = 50$), and pupal weight is highly correlated with final larval weight ($r^2 = 0.91$, $n = 20$). The development of larvae was measured using the BL (mm) and HW (mm) of larvae collected from each system. Generally, the head width capsule was measured because it displays different variations between larval stages (Hsia and Kao, 1987). Panzavolta (2007) used the data of head capsule width and BL to determine instar separation rules, which were analyzed using the Hcap computer program. Developmental studies of insects have also been related to body size evolution, which response to changes in the environmental conditions. For example, environmental parameters such as temperature may affect the development of insects by

changes from their original size at ambient temperature to a smaller size at higher temperatures (Yamada and Ikeda, 2000).

Overall, the developmental size of larvae from all systems showed a growing pattern, except for ORVS F1. The ORVS F1 showed a decrease in the growth rate, with a size difference of about 0.0088 mm. The FACE F1 larvae had the smallest development size at the first 14 days and showed growths in the size of 0.0252 mm. Samples from the RR F1 (0.0111 mm) also showed an increase in the growth rate and final size. These findings support the hypothesis that CO₂ concentration is able to inhibit larval growth (cited). However, further experiments using prolonged exposures of the gas on the next generations of larvae are needed to show a more pronounced effect.

Previous study done by Harrison et al. (2006) showed that hypoxia or low oxygen levels affected the development of *T. molitor*. Ambient P_{O_2} (AP_{O_2}) values of 10 kPa or below resulted in a decrease in the species size. The body size of living organisms may be reduced under hypoxic conditions as these conditions signal the deterioration of environmental conditions and require rapid maturity and small body size for ecological success. High CO₂ concentrations can also affect insect respiration; for example, *Cryptolestes turcicus* shows increased phosphine consumption when the CO₂ level is increased (Ren et al., 1994).

In the present study, *T. molitor* showed a longer development time following exposure to high concentrations of CO₂, especially in the ORVS system, where development took 62 to 69 days longer than for the RR F1. Similar results were obtained from a study on cowpea bruchids by Cheng et al. (2012). Low CO₂ concentration exposure (10% O₂ + 10% CO₂) caused less developmental delay than was observed at a higher CO₂ level (2% O₂ + 18% CO₂). Recently, strong evidence was reported showing that high levels of O₂ have no effect on large insects, as oxygen delivery does not become more challenging in larger insects. A longer delay in the development time of cowpea bruchids is expected with a longer exposure of CO₂. However, opposite results were reported for *Anagasta kuehniella* in which development from the egg-to-adult period was not influenced by CO₂ (Junior and Parra, 2013).

Apart from CO₂, other parameters should also be studied to determine their direct impact on the development of *T. molitor*. For example, the temperature in the ORVS and FACE exceeded the optimal temperature suitable for *T. molitor* reproduction. According to Fiore (1960), the reproduction of *T. molitor* is very appropriate at their optimal temperature of about 25-27.5 °C. The total developmental time for this species is 80.0-83.7 days (Park et al., 2012). At a temperature of 25 °C, a decrease in humidity had no effect on adults, larvae or pupae of *T. molitor*, but it increased mortality at 10 °C (Punzo and Mutchmor, 1980). Other insects, such as the peach fruit moth, *Carpocapsa sasakii*, show temperature dependence in their development processes. The development time of *C. sasakii* eggs was increased by decreasing the temperature below 32 °C, whereas the developmental rates of both larvae (< 28 °C) and pupae (< 32 °C) were faster at the optimum temperature (Kim et al., 2001).

Neighbour-joining (NJ) analysis applied in PAUP 4.0 was used to investigate the associations among taxa in the profiles according to Kumar et al. (2001). The subsequent classification of 'test' taxa is also important because of its strong track record in large species assemblage analysis (Kumar and Gadagkar, 2000). This method has the extra benefit of generating results faster than alternatives (Hebert et al., 2003). The COI gene is one of the most preferred for evolutionary time depth studies because of the ability to conserve protein-coding genes in the animal mitochondrial genome

(Brown, 1985). Moreover, the *COI* primer is very important in the studies of systematics metazoan invertebrates, including acoelomates and pseudocoelomates (Folmer et al., 1994).

Nucleotide changes in DNA sequences were used to create a NJ tree (Fig. 4). The NJ analysis using *COI* sequences revealed ORVS F1 and RR F1 formed in different clades (A and B), with FACE F1 located in both clades. The results showed that the nucleotides in the DNA sequences of *T. molitor* larvae changed after exposure to CO₂ concentrations higher than ambient. The most significant changes were observed in ORVS F1 larvae due to the closed system state, which allowed CO₂ gases to surround the insects and resulted in a higher exposure to CO₂ in the larvae of *T. molitor* than was experienced in the FACE (open) system.

A further difference between the FACE system and ORVS is that the FACE system has been built to expose plants to increments of atmospheric CO₂ with minimal alterations of the natural environment in which the plants are naturally growing. Even so, short-term fluctuations in CO₂ concentration and a substantial infrastructure presence are problems inherent with the FACE system. The FACE system also experiences some experimental restrictions, including the unavoidable presence of CO₂ concentration gradients along the wind direction (Miglietta et al., 2001). The instability displayed by this system, therefore, becomes a factor in the reduction of CO₂ impacts on the sample. For this reason, the system itself could be a major factor affecting the distribution of FACE samples, including the appearance of FACE F1 in both Clade A and Clade B in the NJ tree.

CO₂ effects on *T. molitor* larvae in the FACE system were found in both clades of the NJ Tree. This result can be related to the location of the FACE system itself in the forest area. A forest habitat with a large number of plant species that use CO₂ in photosynthesis will reduce the CO₂ levels in FACE system and automatically decrease the exposure of CO₂ by *T. molitor* larvae. A FACE system built in a field area, as described by Scherber et al. (2013), might be more suitable for use in conducting research on the impact of CO₂ on insects. Less absorption of CO₂ by plants will increase the chances that the larvae will be exposed to higher concentrations of CO₂.

Conclusion

High levels of CO₂ in the environment have the ability to affect the development and life cycle of *Tenebrio molitor*. Increments in CO₂ cause a reduction in larval development and change the nucleotides in the DNA sequences of the mitochondrial *COI* gene. A strong positive correlation coefficient was shown in samples from the rearing room system (RR F1), and moderate or weak positive linear correlation coefficients in larvae from the FACE system (FACE F1) and the ORVS (ORVS F1) (significant at the 99% confidence level). A negative correlation coefficient was found for head length with a head width and body width in both the ORVS and FACE system. These results confirm that the *T. molitor* is able to survive by changing its genetic sequences and that it can adapt to climate changes through evolution. The results of this study clearly show a direct relationship between elevated CO₂ and the decrease in larval developmental size, the analysis demonstrated the occurrence of nucleotide changes in certain individuals of *T. molitor* in response to increased CO₂. The CO₂ concentration can therefore be seen to affect the morphology of *T. molitor*, even though the changes are not drastic and take more than one generation. Further study should be conducted in

the future to increase our understanding of the direct impacts of climate change, including the increase of greenhouse gases, on animals and plants, and specifically on humans. As a suggestion, the next experiments should be conducted to determine the implications of elevated CO₂ towards *T. molitor* development on different generations.

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