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Respiratory activity of the pallid emperor Moth, *Cirina forda* (Lepidoptera: Saturniidae) during larva and pupa developments

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Abstract

Respiratory activity is an important indicator of life processes and physiological status of all organisms. This is because it reveals how energy is acquired, transformed, allocated, utilized or stored in organisms. Estimating metabolic rate in insects therefore provides a viable approach to determining several physiological landmarks such as diapause development. This study investigated the phenomena of diapause in the larval and pupal developmental stages of *Cirina forda* through a measure of its developmental metabolic rates. A total of 36 larvae and 80 pupae were used for the experiment. The insects were reared and maintained under ambient temperature and relative humidity (25-32°C; 75-85%RH) in the laboratory. Metabolic rate was progressively measured as individual's rate of O₂ consumption and CO₂ evolution using spirometric and titrimetric methods respectively over the six larva instar stages and the ten months of pupa developments. Data obtained were compared using Analysis of Variance (ANOVA) and means were separated using Fisher's Least Significant Difference (LSD). The result showed that there was significant suppression of about 87% in O₂ consumption (F_{1, 14}=33.34; ANOVA, P<0.05) and about 86% suppression in CO₂ evolution (F_{1, 14}=9.22; ANOVA, P<0.05) during pupa development. However, the respiratory quotient (RQ) of larva and pupa were not significantly different (F_{1, 14}=4.15; ANOVA, P>0.05). The study revealed that *C. forda* experienced an extremely low metabolism during pupa development which suggested diapause development in the insect and this period of highest metabolic suppression occurred in pupae of age between 90-150days.

Keywords: Diapause, *cirina forda*, metabolic suppression, spirometry, respiratory quotient

Introduction

The class insect has successfully colonized almost all ecological terrains through magnificent development of adaptive strategies. Insects such as the Antarctic Springtail, *Gomphiocephalus hodgson* are found in the Antarctic continent^[34], the goliath beetle, *Goliathus giganteus* in the tropical African forest and the ocean skater, *Halobates germanus* live in the Indian and Pacific oceans^[27]. They have remained one of the most successful of all animals because of their ability to respond adequately to seasonal and environmental changes. Many of these adaptive strategies involve morphological, behavioural, biochemical, molecular and physiological transformations or readjustments.

There are two main strategies used by insects in responding to environmental stress such as temperature, moisture or food availability. They either enter into a reversible state of suppressed metabolism known as quiescence or into a genetically determined obligatory or facultative state of developmental and metabolic suppression known as diapause^[28]. Diapause is a response to seasonal predictive habitat deterioration^[18] and could occur at the embryonic, larval, pupal or adult stage of a given species. Unlike quiescence, diapause is multifaceted preemptive tool used for survival; it usually precedes adverse conditions and does not necessarily end immediately favourable conditions return^[3]. Diapause as a type of dormancy could be during winter (hibernal) or during summer (aestival). The latter enables insects to survive high temperature and it is otherwise referred to as "summer diapause"^[22], "tropical diapause"^[6] or "hot thermal diapause"^[24].

Diapause has been shown to have three eco-physiological sub-phases of induction, maintenance and termination^[20]. Each of these stages is characterized by a number of physiological, biochemical and morphological features^[39].

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One hallmark of diapause initiation is regulated decrease in metabolism^[14, 42]. True diapause is exemplified by the maintenance phase and it is characterized by a period of relatively low and constant metabolic rate which always precedes diapause termination^[20]. Metabolic rate decreases because all metabolically expensive biochemical and physiological systems and processes are shutdown or reduced during diapause^[14]. Examples of such physiological alterations include reduced neurone excitability^[41], cell cycle arrest^[38], reduced cytochrome oxidases activity^[31] and metabolic depression^[29]. High diapause intensity was shown as profound metabolic depression of about 90% in diapausing pupal of fleshfly, *Sarcophaga argrostoma*^[8]. During termination, there is a decrease in diapause intensity; resumption of direct development and most importantly rise in metabolic rate which is an index of diapause completion^[20]. Such rise in metabolic rate may be steep as reported in older diapausing pupal of *Pieris brassicae* and diapausing pupal of flesh fly *S. crassipalpis*^[17, 36].

Metabolism constitutes a fundamental characteristic of all organisms because it remains a means of transforming energy and materials to support life functions and structures^[13]. As part of surviving strategies, many insects can alter their metabolic rate in response to extrinsic or intrinsic factors. Temperature and mass remains the most influential factors on metabolism. Other factors influencing insects' metabolism include age and ontogeny^[10, 45], gender^[40] as well as activity^[13]. A number of insects suppress metabolic rate and enter a hypometabolic or dominant state known as diapause^[12, 15]. Estimating metabolic rate can therefore presents a holistic measure of an organism "pace of life" because metabolism is highly related with many other organisms' physiological status such as diapause. Among several methods of qualifying metabolic rate of an organism; oxygen consumption is technically easy and most satisfactory especially for pupal insects^[11, 39].

Materials and methods

Study animals and rearing

Cirina forda larvae (1st instar) were collected by hand from the host plants *Vitellaria paradoxa* in the field at Km 51 Bida-Mokwa road, Kutigi (Latitude 9° 10'N; 9°, 13' and Longitude 5°35'E; 5°, 39'), Lavun LGA, Niger State, Nigeria in the 4th week of July 2013, 2014, 2015 and 2016. They were brought to the Zoology Department laboratory, University of Ilorin in a perforated paper carton. They were immediately transferred into a culturing wire netted cage of size 100cm x 60cm x 45cm filled with loamy soil to a height of 45cm. The soil was to provide substrate for pupation. Larvae were maintained under ambient temperature and relative humidity (25-32°C, 75-85% RH) and were daily supplied with fresh twigs of *V. paradoxa*.

Metabolic rate measurement

Oxygen consumption of larvae through instar I-VI was measured using respirometric method. Each larva was removed from the culturing cage, starved of food for 6-8 hours, weighed on digital analytical balance (200 ± 0.01g) and immediately transferred into respirometric flask. Each larva was suspended in wire gauze over a 2ml 0.57M potassium hydroxide solution in a respirometric flask. The oxygen consumption rate (VO₂) was monitored over a period of 60 minutes using constant volume manometric technique. Six replicates were run for each larva instar.

Pupae were removed from the soil, cleanse of soil particles with brush and weighed on balance. Each pupa was suspended in wire gauze over a 2ml 0.57M potassium hydroxide solution in a respirometric flask. Oxygen consumption was monitored over 240minutes using constant volume manometric technique. Eight replicates were run on monthly basis and over the ten months (September-June) of pupa development. Oxygen consumption was determined by Warburg technique as described by^[21]. The flask constant (K) was found to be between 4.51-4.54 using the formula described by^[23]. The rate oxygen consumption VO₂ was expressed as µlO₂/g/hr.

At the end of each measurement of oxygen consumption, 0.057M dilute hydrochloric acid (HCl) was titrated against the potassium hydroxide solution in the respiratory chamber. The amount of carbon dioxide expelled during each respiratory assay was determined by titrimetric method as described by^[25]. The CO₂ expelled was then expressed as µlCO₂/g/hr. The respiratory exchange ratio (RER) or respiratory quotient (RQ) was determined for each assay as the ratio of VCO₂ to VO₂.

Statistical Analysis

One way Analysis of Variance (ANOVA) was used to test the difference in metabolic parameters such as oxygen uptake, CO₂ evolution and respiratory quotient of pupae and larvae. Means were separated for significant difference by Fischer's Least Significant Difference (LSD) at P < 0.05.

Results

Table 1 shows the mean respiratory measurements in the course of larval development from larval instar one to six (LI-LVI). There was a decline in mass-specific oxygen uptake as the larvae grew from LI to LVI. There were significant differences in oxygen consumptions (F_{5, 30} = 135.1; ANOVA; P < 0.05) and in CO₂ evolutions (F_{5, 30} = 26.7; ANOVA; P < 0.05). However there was no significant difference in their respiratory quotients (F_{5, 30} = 1.58; ANOVA; P > 0.05) across larval instars. The result showed that smaller larvae have relative higher oxygen consumption and higher carbon (iv) oxide evolution. Moreover, the ratio of carbon (iv) oxide evolved to oxygen consumed (RQ) declines with the age and mass of larvae.

Table 1: Mean respiratory measurement of larvae *C. forda* over the period of development

Stage	Mass (g)	O ₂ Consumed (µlO ₂ /g/hr)	CO ₂ Evolved (µlCO ₂ /g/hr)	RQ
LI	0.63 ± 0.18 ^a	1633.88 ± 193.30 ^a	1008.50 ± 265.18 ^a	0.60 ± 0.09 ^a
LII	0.93 ± 0.16 ^b	1344.70 ± 88.99 ^b	716.93 ± 95.60 ^b	0.53 ± 0.06 ^a
LIII	1.82 ± 0.18 ^c	1066.50 ± 39.13 ^c	646.42 ± 108.75 ^c	0.61 ± 0.11 ^a
LIV	2.53 ± 0.24 ^d	952.95 ± 39.16 ^d	541.68 ± 68.08 ^d	0.57 ± 0.05 ^a
LV	3.40 ± 0.27 ^e	734.78 ± 17.32 ^e	394.43 ± 36.92 ^e	0.54 ± 0.04 ^a
LVI	4.56 ± 0.43 ^f	563.70 ± 23.52 ^f	297.73 ± 48.81 ^f	0.53 ± 0.08 ^a
LSD	0.17	55.7	78.9	-

Values are mean of six replicates

Mean carrying the same superscript are not significantly different (ANOVA, LSD, $p < 0.05$)

Table 2 shows mean monthly respiratory measurements of pupae over ten months of development (PI-PX). There was a gradual decline in pupae oxygen uptake over the ten month. Oxygen uptake attained its lowest value at PV (January) before it subsequently rose sharply from February to June (PVI-PX). The respiratory quotients of pupae also declined

with age of the pupae. Generally there were significant differences in oxygen consumptions ($F_{9, 69} = 6.26$; ANOVA; $P < 0.05$), CO_2 evolutions ($F_{9, 69} = 4.79$; ANOVA; $P < 0.05$) and in the respiratory quotients across the ten months of pupa development ($F_{9, 69} = 3.28$; ANOVA; $P < 0.05$).

Table 2: Mean respiratory measurement of dormant pupae *C. forda* over ten months

Stage	Mass (g)	O ₂ Consumed ($\mu\text{O}_2/\text{g/hr}$)	CO ₂ Evolved ($\mu\text{CO}_2/\text{g/hr}$)	RQ
PI	2.90 ± 0.86 ^a	106.94 ± 36.76 ^a	85.26 ± 28.75 ^a	0.83 ± 0.23 ^a
PII	2.82 ± 0.99 ^a	105.56 ± 31.66 ^a	84.95 ± 30.28 ^a	0.81 ± 0.13 ^b
PIII	2.58 ± 0.74 ^{ab}	90.51 ± 24.14 ^a	73.89 ± 21.64 ^a	0.72 ± 0.12 ^c
PIV	2.99 ± 0.91 ^{abc}	86.34 ± 35.68 ^a	68.71 ± 32.40 ^a	0.77 ± 0.11 ^c
PV	2.79 ± 0.86 ^{abcd}	85.85 ± 36.15 ^a	52.84 ± 08.07 ^b	0.61 ± 0.07 ^d
PVI	2.61 ± 0.81 ^{abde}	131.55 ± 65.48 ^{ab}	57.13 ± 29.31 ^{bd}	0.44 ± 0.07 ^e
PVII	1.92 ± 0.66 ^f	166.80 ± 41.69 ^c	89.80 ± 32.81 ^{ac}	0.53 ± 0.13 ^f
PVIII	1.91 ± 0.65 ^f	181.33 ± 40.55 ^d	102.35 ± 25.65 ^f	0.57 ± 0.07 ^{fg}
PIX	2.32 ± 0.84 ^{fg}	225.69 ± 129.99 ^e	135.82 ± 76.14 ^g	0.62 ± 0.11 ^{gh}
PX	2.22 ± 0.46 ^{hi}	232.14 ± 18.29 ^{ef}	116.86 ± 26.26 ^{gh}	0.51 ± 0.11 ^{ghi}
LSD _{0.05}	0.29	27.24	14.64	0.06

Values are mean of eight replicates

Mean carrying the same superscript are not significantly different (ANOVA, LSD, $p < 0.05$)

Table 3 shows the average oxygen consumption of larva and pupa. The mean respiratory rate showed that mass-specific oxygen uptake of pupae declines by about 87% while that of carbon dioxide evolution falls by about 86%. There was a significant difference between average oxygen uptakes in $\mu\text{O}_2/\text{g/hr}$ ($F_{1, 14} = 33.34$; ANOVA, $P < 0.05$) of pupae and larvae. There was also a significant difference in CO_2 emission in $\mu\text{CO}_2/\text{g/hr}$ of pupae and larvae ($F_{1, 14} = 9.22$; ANOVA; $P < 0.05$). However, the respiratory quotient of pupae and larvae were not significantly different ($F_{1, 14} = 4.15$; ANOVA; $P > 0.05$). Similarly the mean mass of pupae and larvae used were not significantly different ($F_{1, 14} = 0.34$; ANOVA; $P > 0.05$). This implied that the respiratory metabolism of *C. forda* pupae was depressed about 7 folds when compared to that of larvae.

Table 3: Mean respiratory measurement comparison of pupae and larvae *C. forda* over the period of development

Stage	Mass (g)	O ₂ Uptake ($\mu\text{O}_2/\text{g/hr}$)	CO ₂ Expelled ($\mu\text{CO}_2/\text{g/hr}$)	RQ
Pupae	2.51 ± 0.39 ^a	141.24 ± 1.18 ^a	86.76 ± 26.01 ^a	0.64 ± 0.13 ^a
Larvae	2.31 ± 1.50 ^a	1049.36 ± 93.17 ^b	600.94 ± 252.87 ^b	0.56 ± 0.04 ^a

Mean carrying the same subscript are not significantly different (ANOVA; LSD; $P < 0.05$)

Fig 1 shows the pattern of oxygen consumption across larva (July and August) and pupa (September to June) stages. The oxygen consumption of larvae were very high an indication of higher metabolism. This suddenly fell at the beginning of pupa development in September till January where it attained its lowest value before slightly risen through to the month of June as the insects exit dormancy. The shape of the curve represents more or less a U-shape trend

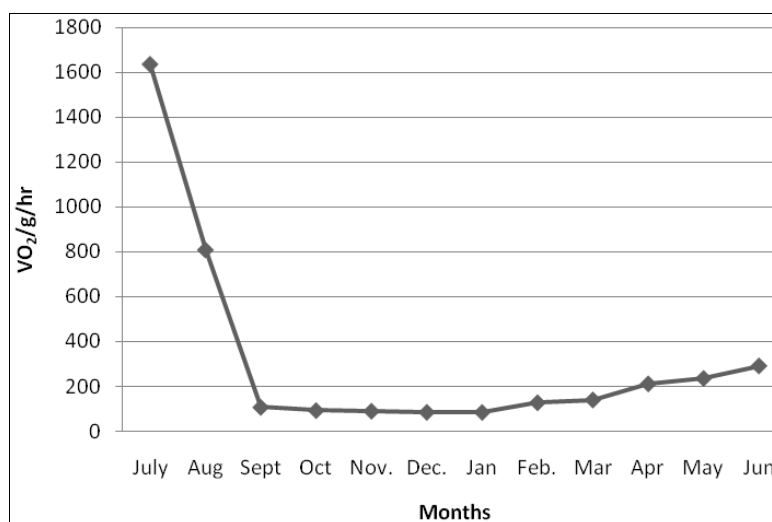


Fig 1: Respiratory patterns in *C. forda* larvae and pupae

Discussion

The rate of oxygen consumption was used as a measure of

metabolic rate in larvae and pupae *Cirina forda* in this study. Absolute oxygen consumption was found to increase as the

larvae developed. However mass-specific oxygen consumption declines as the larvae develop or as they increase in size through instar I to instar VI with mean oxygen uptake of about 1049.36 $\mu\text{O}_2/\text{g}/\text{hr}$. This value is approximately close to 920 $\mu\text{O}_2/\text{g}/\text{hr}$ reported for larvae *Bombyx mori* [4]. Decrease in mass-specific oxygen consumption has also been reported in larvae *Manduca sexta* [32] and *Bombyx mori* larvae [5]. In *Bombyx mori* larvae, a decrease metabolic demand with age (mass) has been shown to be correlated with a decrease in cytochrome oxidases activities [5].

Oxygen consumption of dormant pupae *C. forda* declined gradually until minimum in the first five months of dormancy and rises gradually thereafter from the sixth month till adult emergence. The average mass-specific oxygen consumption during the entire period of pupae dormancy was about 141.24 $\mu\text{O}_2/\text{g}/\text{hr}$. This value was about 87% lower than the average value of oxygen consumption in the entire larval development. Similar species-dependent metabolic depression has been reported in other dormant insects. For instance a 15% metabolic depression was reported in Monarch butterfly [7] while about 90% depression in metabolism was reported in diapausing pupae of Flesh fly [8, 29]. Metabolic suppression is a strategy for saving metabolic cost in diapausing insects [15]. Many a times, metabolic depression is preceded by a careful selection of diapausing site in order to reduce metabolic stress [16, 34]. Such site selection was noticed in wandering 6th instar larvae *C. forda* before they eventually dig into the soil for pupation.

The mean respiratory quotient (RQ) of larvae *C. forda* ranged between 0.53 to 0.60, while mean monthly respiratory quotient of dormant pupae ranged between 0.44 and 0.83. In both groups, the values of respiratory quotient decreases with the age of the insect. However, the average respiratory quotient of larvae and pupae were not significantly different. The average monthly respiratory quotient of 0.64 ± 0.11 obtained for dormant pupae *C. forda* was however lower than 0.78 ± 0.09 and 0.72 ± 0.09 respectively reported for intact and brainless *Cecropia* silkworm [33] and 0.70 reported for diapausing pupae of *Hyalophora cecropia* [36]. However this average RQ of dormant *C. forda* was higher than 0.10 to 0.30 reported for diapausing pupae of *Phalera and Endromis* [1, 2]. The average RQ of 0.64 obtained for dormant *C. forda* seems to be a valid indication of the type of metabolism the insect undergoes at this stage of its development and it probably signifies the combustion of mixture of fats, proteins and carbohydrates as its respiratory substances.

The pattern of curve in fig.1 is in agreement with those reported by [33] for diapausing pupae of *Cecropia* silkworm. The months of dormancy in pupae *C. forda* (November to January) are characterized by an extremely low metabolic rate of about 91% and such corresponds to the horizontal portion of the U-shape curve of diapause metabolism. This U-shaped curve of metabolism shows the metabolic characteristics of biphasic periods of rapid rearrangement and maximum morphogenic changes. During the first phase, that is at beginning of the last larval instar to pupa transformation, the respiratory metabolism decreases precipitously by about 82% and then declines gradually until its minimum in January. The second phase presented an initial surge of about 35% rise in metabolism after a preceding maximum decline. Metabolic rate thus gradually continues to rise until adult emergence. This type of respiratory pattern has been used to describe respiratory landmarks of diapausing insects [43] which include

a precipitous drop in metabolic rate as the induction, steady metabolic decline as the maintenance and precipitous increase in metabolic rate as the termination phase [35]. With all these recognizable stages in pupa *C. forda*, it implied that the insect undergoes diapause at certain period of its pupa development. Precisely diapause was most likely occurring between the 3rd and 5th months of pupation development (November to January).

Normal respiratory quotient (RQ) values in animals ranges from 0.70 (indicating fat metabolism) to 0.82 (indicating protein metabolism) and 1.0 (indicating carbohydrate metabolism). However, RQ in some insects can fall below 0.70 during synthesis of carbohydrate via gluconeogenesis [19] or above 1.0 during the synthesis of fat from carbohydrate [19, 26]. When RQ falls below 0.70 in insects, such state is often compared to a period of hibernation in mammals [44]. Therefore respiratory quotient observed in larvae *C. forda* could be attributed to the process of gluconeogenesis probably as a result of the 6-8 hours of food deprivation before respiratory measurement commences, while the RQ recorded in dormant pupae of *C. forda* could be attributed to diapause development.

Conclusion

During *C. forda* development, there was significant suppression in oxygen consumption and carbon dioxide evolution at the pupa stage compare to larva stage. The period of this critical suppression was between the months of November to January when the insect was between 3-5 months into pupa development. The respiratory attributes at this period indicates that the insect was actually undergoing diapause at such period of extreme respiratory depression. The insect showed the respiratory landmarks of a precipitous drop in metabolic rate as the induction, steady metabolic decline as the maintenance and precipitous rise in metabolic rate as the termination phase.

Conflict of interests

The authors have not declared any conflict of interests.

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