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Thank you for your assistance.
Enhancing bio-suppression of *Parthenium hysterophorus* L.: Diapause in *Zygogramma bicolorata* Pallister and its manipulation through insulin-like peptides (ILPs)

R.K. Gupta a,⁎, Salil Gupta b, K. Bali a, K. Srivastava a

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A B S T R A C T

In-depth investigations on diapause behaviour of *Z. bicolorata* revealed that the adults entered diapause at any time from August to December and that the number peaked (42.00%) during the last half of November. The percentage of adults entering diapause increased with a decrease in day length. Weight of diapausing adults was significantly higher than weight of non-diapausing adults. The percentage of adults undergoing diapause at 30 °C was significantly lower than those undergoing diapause at 15 and 25 °C. The percentage of adults burrowing increased with increasing moisture. In silty soil and soil with high organic matter, 46.7% and 49.2% of adults entered diapause, respectively, whereas in sandy soil, only 23.5% of adults entered diapause. When newly emerged beetles were exposed to 5 μg of human insulin 30/70, a significantly lower percentage of treated adults underwent diapause compared to untreated adults under both feeding and no feeding conditions. Insulin treatment also influenced the emergence period from diapause (93.92±1.73 days), percent emergence (81±1.54%) and fecundity/month ($127.7±25.38$ eggs) of *Z. bicolorata* in treated adults as compared to untreated adults (109.05±2.2, 74.00±1.82 and 438.3±19.33 eggs, respectively). However, there was no significant impact of insulin on adult longevity. These findings are of great utility in the biological suppression of *Parthenium* as it will enhance the effectiveness of this beetle through manipulation of diapause.

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R.K. Gupta a,⁎, Salil Gupta b, K. Bali a, K. Srivastava a

Introduction

Biological control of *Parthenium hysterophorus* L. (Asteraceae) is the most cost-effective, environmentally safe and ecologically viable alternative management strategy. A biocontrol program of this weed was initiated in India in 1983 with the introduction of the leaf feeding beetle, *Zygogramma bicolorata* Pallister (Chrysomelidae: Coleoptera). The establishment of the beetle resulted in a significant reduction of *P. hysterophorus* in localized areas. Further biocontrol programs were initiated in 1989 in Jammu and Kashmir and large scale releases were made in 1992 (Gupta et al., 2002). Since then, the beetle dispersed over an area of more than 9000 km² (Gupta, 2008) and weed suppression is evident in many parts of the state (Gupta et al., 2004).

Introduced insects do not always get to live in an environment that makes their optimal growth possible. They must adapt to and survive conditions that do not support continuous development. Diapause plays an important role in the life cycle of this beetle as an adaptive strategy to escape adverse seasonal conditions (Jayanth and Bali, 1993a). *Z. bicolorata* adults burrow into the soil and diapause within chambers that they form approx. 1 to 2 cm beneath the surface. They enter diapause when conditions are unfavourable, especially in the regions where day length and temperature differ within a season (Tauber et al., 1986). The egg, larval and pupal stages do not enter diapause. Non-diapausing adults are capable of breeding under laboratory conditions during winter. Normally, this insect remains active between March and October and, after completely defoliating *Parthenium* plants in a particular area, migrates to new areas. As a result, the end of the rainy season, very few adults are likely to be present in the area of initial defoliation. However, since diapause occurs throughout the breeding season, some diapausing adults are left behind at each location, which are ready to defoliate the growth of the weed during the following year. This eliminates the necessity of reintroducing the beetle to the same area if the weed density increases (Jayanth and Bali, 1993b). Even though defoliation by *Z. bicolorata* has a visible effect on *Parthenium* under field conditions, the timing of diapause onset, maintenance and termination may determine successful establishment of this beetle to new areas. When diapause is facultative rather than obligatory, insects can develop without interruption while conditions are favourable. In *Z. bicolorata* this phenomenon turns out to be both advantageous as well as disadvantageous. This is an advantage because it can exploit seasonal resources and bridge harsh winters, dry seasons or other
seasonally harsh conditions that would otherwise prove unfavourable for beetle survival. However, since it leads to arrest in development and suppressed metabolism, the diapaused beetle resumes their activity late in the season when the *Parthenium* has germinated and started flowering. The ability of this beetle to synchronize its period of activity to the availability of its plant host is likely to play a major role in its establishment and effectiveness as a biological control agent (Gupta et al., 2006). Therefore, manipulation of diapause may aid in improving the efficiency and efficacy of biological control so that it can terminate early in the season before pre-monsoon *Parthenium* starts flowering.

Several environmental and biological factors influence diapause behaviour of *Z. bicolorata* (Jayanth and Bali, 1993b), but it is difficult to manipulate them under natural conditions. However, it is possible to manipulate diapause using insulin-like peptides (ILP) (Arpagaus, 1987) or insect growth regulators (IGR) (Watanabe and Tanaka, 2000). Insects do not have a tissue that is specialized in carbohydrate homeostasis such as the pancreas. However, they do have a group of insulin-producing cells (IPC) that are located in the brain and constitute an endocrine organ for the regulation of growth and sugar metabolism (Geminard et al., 2006).

After their release into open hemolymph circulation, the *Drosophila* insulin-like peptides reach their target tissues and elicit their biological functions. Insulin signaling mediates JH synthesis and is integral to diapause in the mosquito, *C. pipiens* (Sim and Denlinger, 2008). These insulin signaling pathways and FOXO (forkhead transcription factor) control key characters of diapause, including the metabolic switch to lipid storage, the halt in ovarian development, and enhanced overwintering survival. Under long day lengths, insulin signaling leads to the production of JH which prompts ovarian development, and to the suppression of FOXO which prevents the accumulation of fat stores. By contrast, under short day lengths, the insulin signaling pathway is shut down, which halts JH synthesis and releases the suppression of FOXO. Genes encoding both insulin-like peptides-1 and -5 were suppressed using RNA interference in mosquitoes that were programmed for nondiapause, and ovarian maturation (Sim and Denlinger, 2009). Knocking down insulin-like peptide-1 with RNAi in non-diapausa mosquitoes resulted in a cessation of ovarian development akin to diapause, and this arrest in development could be reversed with an application of JH. Considering all these observations, an in-depth investigation was carried out to understand diapause behaviour of *Z. bicolorata* under varied conditions for better biological suppression of *Parthenium*.

**Materials and methods**

**Natural diapauses**

Burrowing was used as a behaviour indicator for diapause. Studies on diapause in *Z. bicolorata* were carried out in diapause cages. A diapause cage consisted of a clear glass jar (15 cm diameter) with a 5 cm column of lightly moistened, air dried, field collected soil (clay loam) compressed with a wooden rod. The cage was covered on the ventral side with a white muslin cloth tied with rubber bands. One hundred adults were collected from the field every two weeks from 15th of June to 31st of December, 2007. They were fed for one week with *Parthenium* leaves (plants grown in small plastic pots) and then released into four diapause cages (25 adults in each) without food and monitored for one week. Adults which burrowed into the soil within one week were counted as being in diapause. Individual weights of diapausing adults were measured using an electronic balance (Avon weighing system Ltd.).

**Diapause behaviour under controlled conditions**

**Rearing of beetle**

Ten pairs of beetles (7 days old) were released in a glass jar of 30 cm diameter (A) containing a freshly cut, apical *Parthenium* stem placed in a glass vial filled with water. The open mouth of the vial was covered with parafilm so that only leaves were available to adult beetles for feeding and egg laying. After 24 h, the beetles were discarded. They were allowed to lay eggs for 24 h only because we desired the cohorts of grubs of the same age. On day 4, the newly hatched grubs that emerged from the eggs were provided with a fresh vial with a freshly cut, apical *Parthenium* stem. The developing grubs were provided a fresh vial on alternate days until day 15 when the fully matured grubs dropped to the bottom of the jar and burrowed into the soil for pupation. Adults emerged on day 24, however the female started laying eggs on day 31 (7 days pre oviposition). So after every month, rearing cycle of test insect was maintained as described above. Since the diapause is influenced by the age of adults and about 50% diapause was obtained in 60 day old adults (Jayanth and Bali, 1993b), we selected adults of this age from our laboratory population in November. Each treatment consisted of 125 adults (5 replicates of 25 adults) in diapausa cages placed in biological oxygen demand incubators maintained at 70 ± 5% relative humidity and 10 h light period. Each cohort was observed for the extent of diapause.

**Effect of temperature on diapause behaviour of *Z. bicolorata***

To study the effect of temperature, a diapausing cage was filled with clay loam soil compressed with a wooden rod, and the moisture level was adjusted to 15% w/v. For each treatment, five cages with 25 beetles each were then placed in BOD incubators maintained at 15, 25 or 30 °C and observed for percent diapause within one week.

**Effect of soil type on diapause behaviour of *Z. bicolorata***

Beetles were released in diapausa cages containing clay loam soil, sandy soil, silty soil and soil containing >0.75% organic matter. The moisture level for each soil type was maintained at 15% w/v. Adults that burrowed were counted as being in diapause.

**Effect of soil moisture on diapause behaviour of *Z. bicolorata***

To study the role of soil moisture on diapause behaviour of *Z. bicolorata*, beetles were released in diapausa cages filled with oven dried clay loam soil containing 5%, 10% or 25% soil moisture w/v and placed in BOD incubators as above. They were observed for one week for initiation for diapause and the number of adults burrowing was noted.

**Effect of insulin treatment on diapause behaviour of *Z. bicolorata***

Two hundred adults were anesthetized (using CO₂) and were individually given an intra-hemocelic application of 5 μl of human insulin 30/70 (30% soluble insulin/70% Isophane insulin) on the ventral side of the abdomen. The injections were made through the side of the abdomen at the third abdominal inter segmental membrane with a Hamilton microsyringe 10 μl. Another 200 untreated adults (control group) received only carrier solution. Treated and control group (eight replicates of 25 adults each) were released into diapausa cages which were prefilled with clay loam soil. These insects were not provided any food during the experimental period. A similar set of experiment was conducted wherein the adults were provided with *Parthenium* leaves in diapausa cages. All the experimental cages were kept at 26 ± 2 °C, 70 ± 5% relative humidity with a 10 h light period. The numbers of adults burrowing per replicate were observed in each group for two weeks. For post-diapausa studies, in experimental cages where diapausing adults had burrowed earlier, water was sprinkled to study the emergence. The first thirty adults were observed to determine the number of days taken to emerge from diapausa. Ten pairs of post-diapausa adults were selected from each group and reared individually on leaves of *Parthenium* for recording their longevity and fecundity.
All data were checked for normal distribution. Data on natural diapause of beetle was subjected to correlation and regression analysis with meteorological data and day length. Laboratory diapause data were subjected to ANOVA followed by Tukey’s post hoc test for comparison of means. All percentage data were subjected to arcsine square root transformation prior to ANOVA. The Mann–Whitney U test was used to test the significance of the mean weights of diapausing and non-diapausing beetles, as well as for the comparison of diapause in insulin treated and non-treated beetles. All statistical analyses were performed by SPSS 11.0.

**Results**

*Z. bicolorata* adults burrowed into chambers they formed 1 to 4 cm beneath the soil surface. None of the adults collected during June and July in Jammu entered diapause. However, diapause gradually increased from 4 to 37% from August to December (Fig. 1A). The percentage of adults entering diapause increased with time. From August to December when the day length became shorter, this percentage went up and peaked at 42±6% during the last half of November. The day length influenced the diapause behaviour in 96.6% of the cases (Fig. 1B) and can be predicted by a regression equation as

\[ Y (% \text{ diapause}) = 148.830 - 11.0893 \times \text{(day length)} \]

with a coefficient of determination \( (R^2) \) value of 96.6%. Moreover, day length, maximum temperature, minimum temperature and rainfall exhibited a significant negative correlation with percent diapause (Table 1) with correlation coefficients of 0.971, 0.797, 0.962 and 0.554, respectively.

Mean weight of diapausing adults was significantly higher than those of non-diapausing adults in October \((z = -3.592, df = 9)\), November \((z = -2.268, df = 9)\) and December \((z = -3.556, df = 9)\), 2007 (Fig. 2).

**Table 1**

<table>
<thead>
<tr>
<th>Max. temp. °C</th>
<th>Min. temp. °C</th>
<th>Rainfall (mm)</th>
<th>Mean relative humidity (%)</th>
<th>Day length (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diapause %</td>
<td>-0.797**</td>
<td>-0.962**</td>
<td>-0.554**</td>
<td>-0.734***</td>
</tr>
</tbody>
</table>

* Correlation is significant at 0.05 level.
** Correlation is significant at 0.01 level.

![Fig. 1. A, Natural diapause in *Z. bicolorata* and its relationship to day length under Jammu condition. B, Regression plot showing influence of day length on diapause behaviour of beetle.](image-url)
Diapause behaviour under controlled temperature

Temperature significantly influenced adult diapause \((F=7.355; df=14, P<0.05)\). The percent diapause decreased significantly from 40.8 to 20.8% with the increase in temperature from 15 to 30 °C \((\text{Table 2})\).

Role of soil moisture and soil type on diapause behaviour of Z. bicolorata

\(Z. \text{ bicolorata}\) adults attempted to burrow into dry soil but were not able to penetrate. However, when the soil surface was moistened, the beetle started burrowing in the soil. The percentage of adults entering diapause increased from 8.8 to 47.20 with the increasing moisture level from 5.0 to 25.0% \([F=22.78, P<0.05]\) \((\text{Table 3})\). The highest diapause percentage (49.2%) was observed in soil with organic matter >0.75% and lowest diapause percentage (23.5%) was observed in sandy soil \([F=5.55, P<0.05]\) \((\text{Table 4})\).

Effect of insulin treatment on diapause behaviour of Z. bicolorata

Topical application of 5 μg of human insulin 30/70 caused \(Z. \text{ bicolorata}\) adults to undergo diapause at a significantly lower percente (21%) than untreated adults (37.5%), when kept in diapause cages without feeding \((z=-3.784, df=7, P<0.05)\). Even when the diapasing beetles were allowed to feed on \(Pallister\) in diapasing cages, the percent diapause was significantly lower (16.5%) in treated adults compared to untreated adults (29.0%), \([z=-3.804, df=7, P<0.05]\) \((\text{Fig. 3})\). Further, insulin treatment significantly influenced emergence period from diapause, percent emergence and fecundity (per month) of \(Z. \text{ bicolorata}\) which were 93.92 ± 1.73, 81.1 ± 1.54 and 5127 ± 25.38, respectively, in treated adults compared to 109.05 ± 2.22, 74.1 ± 1.82 and 4383 ± 19.33, respectively, in untreated adults. However, there was no significant impact of insulin on the longevity of adults.

Discussion

The present study has clarified the role of certain environmental and biological factors on the diapause behaviour of \(Z. \text{ bicolorata}\). However, diapause itself is not a well defined state, but a dynamic succession of developmental, behavioural and physiological events \((\text{Taubert et al., 1986})\). Diapause behaviour of \(Z. \text{ bicolorata}\) is complex. Not all adults of \(Z. \text{ bicolorata}\) enter diapause and it is unclear whether diapause is obligatory. At the same time, this, too, could not be ascertained what triggered diapause behaviour in \(Z. \text{ bicolorata}\), especially because abundant food was available and weather conditions were favourable from July to October which rules out the possibility of this being facultative diapause. \(J. \text{ Pallister and its manipulation through insulin-like peptides (ILPs), J. Asia Pac. Entomol. (2010), doi:10.1016/j.aspen.2010.06.006}\) 

![Fig. 2. Mean adult weight in diapasing and non-diapasing population of Z. bicolorata during peak period of diapause.](image)

![Fig. 3. Effect of insulin on diapause behaviour of Z. bicolorata.](image)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>No. recovered after 7 days</th>
<th>Per cent diapause</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 °C</td>
<td>125</td>
<td>20.8a</td>
</tr>
<tr>
<td>25 °C</td>
<td>125</td>
<td>28.6b</td>
</tr>
<tr>
<td>15 °C</td>
<td>123</td>
<td>40.8c</td>
</tr>
</tbody>
</table>

\*Mean within a column superscripted by different letters are significantly different \((P<0.05, \text{Tukey HSD})\).
**Calculated by averaging 5 replicates of 25 diapausing adults.

<table>
<thead>
<tr>
<th>Type of soil</th>
<th>Per cent diapause</th>
</tr>
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<tbody>
<tr>
<td>Sandy soil</td>
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<tr>
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<tr>
<td>Soil with organic matter (&gt;0.75%)</td>
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Table 3

<table>
<thead>
<tr>
<th>Per cent soil moisture</th>
<th>Per cent diapause</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8.8a</td>
</tr>
<tr>
<td>10</td>
<td>26.4b</td>
</tr>
<tr>
<td>25</td>
<td>47.20c</td>
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**Calculated by averaging 4 replicates of 25 diapausing adults.
reported similar findings for inducing diapause in Zygogramma
sauralis. However, in the Colorado potato beetle, another related
insect, pupae or new adults were the most vulnerable for diapause
with a critical photoperiod of 15 h at 25°C (de Kort, 1990). The
criterion of burrowing behaviour as an indicator of diapause was
originally used and tested against Z. bicolorata by Jayanth and Bali
(1993b) and in the Colorado potato beetle by Bech (1968), de Wilde
(1969), and de Wilde and Hsiao (1981). Adults were capable of
entering diapause after burying themselves 1 to 6 cm into the soil,
which probably makes it a more efficient biological control agent
because it eliminates the necessity of reintroducing the beetle to the
area where it was originally introduced and also because it ensures
proper colonization and dispersal of this beetle (Jayanth and Bali,
1993b). While the burrowing adults are able to consume the recurrent
growth of the weed during the following year, they resume their
activity late in the season when the Parthenium germinates and starts
flowering. Our past studies reveal that even though defoliation by Z.
bicolorata has a visible effect on Parthenium under field conditions, the
timing of onset, maintenance and termination of diapause are
important in the successful adoption of this beetle to new areas.
Since Parthenium seeds persist in the soil for a long time with about
50% of the buried soil seed-bank remaining viable for up to 6 years
(Navie et al., 1998). It continues to germinate throughout the year
from the accumulated soil seed-bank and many flushes occur
throughout the year. Five such major germinating flushes of P.
hysterophorus have been observed in one year. Defoliation by Z.
bicolorata had significant impact on plant height, plant density and
flower production in flushes #1 and #2 but it did not lead to any
significant influence in flushes #3 and #4. These two flushes exhibited
longer periodicity, profuse branching, a longer flowering period and
maximum flower production and contributed the majority to the
existing seed soil bank in the area of study. However, the appearance
of the beetle on these two flushes was delayed and asynchronous.
Because these flushes contribute less to the soil seed-bank, the
existing soil seed-bank is unlikely to be depleted in totality. Therefore,
either the augmentative releases of laboratory reared beetles should be
carried out during March–April every year on these flushes or
beetle diapause should be manipulated to facilitate its early
termination and to synchronize it with the early flushes, #1 and #2.

The percentage of field collected adults burrowing into the soil
increased from August to November in Jammu. This increase in
diapause was attributed to decreased day length, decrease in
temperature and senescing host–plant foliage, as reported in L.
decemlineata by Hare (1990). The reduction in percentage of diapause
observed during October could be due to the presence of many non-
diapauing adults from previous generations and the dryness of the soil
(rainfall during October was 0 mm and R.H. ranged from 59.5 to
64.5%). These results substantiate earlier findings of Jayanth and Bali
(1993a). As the percent diapause of Z. bicolorata increased from
August to December, the light period decreased from 13.16 to 9.87 h,
which negatively influenced diapause behaviour of Z. bicolorata in
96.6% of the insects. Tauber and Tauber (1976) observed that
photoperiod plays an important role in the induction and termination
of diapause in Leptinotarsa decemlineata, a very closely related beetle
which entered diapause after exposure to a critically short photoperi-
od. This short day diapause is probably a complex of physiological
regulatory mechanisms at the neuro-endocrinological and target
tissue levels (Tauber et al., 1988). While inactivation of the corpora
allata remained one feature of the diapause syndrome, changes in
ecdysteroid concentration in the Colorado potato beetle in relation to
day length were also observed and it was suggested that ecdysteroids
might be involved in induction and termination of diapause (Briers
and de Loof, 1981; Briers et al., 1982).

Mean weight of diapausing adults was significantly higher than
those of non-diapausing adults during the peak period (October,
November and December). This indicates that diapausing and non-
diapausing individuals can be differentiated on this criterion. This
higher mean weight of diapausing adults might be due to the
accumulation and breakdown of metabolic reserves, such as lipids and
glycogen (Tauber et al., 1986; Danks, 1987; Adedokun and Denlinger,
1984), the deposition of extra layers of hydrocarbons for water
proofing the cuticle (Yoder et al., 1992), or the synthesis of storage
proteins (Chippendale, 1988). In Colorado potato beetles, food
utilization during diapause was used primarily to increase nutritional
reserves in the fat body and not for reproduction. Lipids started to
accumulate in the fat body immediately after emergence, followed by
proteins from day 6 onwards (Dortland and de Kort, 1978). The
proteins, which accumulated first in the haemolymph and later in the
fat body, have been called diapause proteins. Also, other blood
proteins increased under short day, resulting in a concentration of
total proteins 3 times higher in the haemolymph (de Kort, 1990).

In the present study, the experiment was planned with a
temperature range of 15 to 30°C, as continuous exposure to 30°C or
higher induced emergence in this pest within few days (Jayanth and
Bali, 1993c). A lower percentage of adults underwent diapause as the
temperature increased from 15 to 30°C. The significance of this inverse
relationship between temperature and diapause can be utilized for
our future studies on comparing diapause among intra-population or
for identifying a non-diapauing strain through laboratory selection.

Over the last ten years, there has been heavy defoliation by this beetle
in moist areas near streams and canals. Therefore, it was necessary to
certain whether this occurs due to soil moisture. Our findings clearly establish that soil moisture plays an important role during
diapause initiation and emergence. It provides ideal physical condi-
tions for successful burrowing and emergence from diapause chambers. Moreover, unseasonal rains during November and December
may provide beneficial conditions for burrowing. Jayanth and Bali
(1993b) observed that Z. bicolorata adults could not penetrate dry soil
for diapause and that they attempted burrowing but were unable to
penetrate when the soil alone was moistened. Most of the adults
collected during November, when the soil was dry, burrowed within
2 h of being given moist soil in the laboratory. In moist areas, as the
proportion of adults undergoing diapause remains high, more adults
emerged from the soil after termination of diapause. More defoliation
was then observed. The physio-chemical attributes of soil, such as
structure, texture, and water potential, also played an important role
in diapause of Z. bicolorata. While information on this aspect in the
target species is completely lacking, this phenomenon is well known in the Colorado potato beetle. Canal soil (silty) and soil with high
organic matter (>0.75%) were most suitable for diapause. Lighter and
loamy soil rich in organic substance with better aeration and no
excessive water were most suitable for Colorado potato beetle
(Kuusik et al., 2001; Hiesaar et al., 2006). Significant variation in
percentage of adults undergoing diapause in beetles collected from
different geographical populations suggests that the beetle is
gradually adapting to adverse conditions in different locations.
Therefore the scope for selection of non-diapauing strains of this
beetle in the future cannot be ruled out. As cooler areas lead to
increased diapause, the beetle is likely to have more impact in areas
with moderate climate.

Insulin-like peptides (ILPs) exist in insects and are encoded by
multigene families that are expressed in the brain and other tissues.
Upon secretion, these peptides likely serve as hormones, neurotrans-
mitters, and growth factors. Suppression of insulin signaling has been
implicated in the induction of adult diapause in D. melanogaster
(Williams et al., 2006; Tu et al., 2005) and is integral to diapause in the mosquito, C. pipiens (Sim and Denlinger, 2009). Our findings suggest that insulin plays an important role in the termination of diapause,
along with various physiological and biochemical changes, without
affecting longevity or fecundity of Z. bicolorata. Further, treated adults
exhibited a low percentage of diapause, reduced time for emergence
from diapause and increased percent emergence. The ability of

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vertebrate insulin to break pupal diapause in *Pieris brassicae* is well documented. It triggers a reactivation of the neuroendocrine system leading to a neosynthesis of ec dysone beginning 6 days after treatment (Arpagaus, 1987). The involvement of ec dysone in the induction and termination of diapause is well established in adults of CPB (Briers and de Loof, 1981; Briers et al., 1982). Watanabe and Tanaka (2000) showed that topical application of pyriproxyfen to *Aulacophora nigripennis* adults led to early termination of diapause, but also changed various physiological and biochemical traits such as chill tolerance and myo-inositol, lipid and glycogen metabolism. This suggests that most of the overwintering traits of this bee may be under the control of JH which generally regulates adult reproductive diapause (Danks, 1987), which was also the case for the beetle (Watanabe and Tanaka, 1998). Cold-hardiness and lipid metabolism were often linked to the diapause in insects that have winter diapause (Denlinger, 1991). The potential role of insulin or ec dysoids for enhancing the efficacy of *Zygogramma* beetle on *Parthenium* control cannot be ruled out and will be explored further.

**Q8 Uncited reference**

Wu & Brown, 2006

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