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Expression of regucalcin gene in cold acclimated diapause eggs of red cotton bug *Dysdercus cingulatus* (Fabricius, 1775)

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Abstract
The expression of regucalcin gene in cold acclimated diapause eggs of *Dysdercus cingulatus* was studied. The DNA and RNA were isolated from diapause induced eggs to low temperature and were used for the amplification of regucalcin gene. The PCR gel electrophoresis image shows that RNA and DNA obtained from the eggs had no traces of regucalcin gene. Thus present study shows that regucalcin gene was not expressed in diapause eggs and it may not play any role in cold tolerance diapause eggs.

Keywords: regucalcin gene, cold acclimation, diapause and *Dysdercus cingulatus*

Introduction
In nature, organisms exposed to changing temperature generally inflict stress which may result in the evolution of adaptive genetic mechanisms to cope up with extreme temperatures (Dillon, *et al.*, 2009; Ayrinhac, *et al.*, 2004 and Rako and Hoffmann, 2006 [7, 2, 17] there are limited studies that support the involvement of genes for survival at low temperature in insects, (Clark and Worland, 2008) [5].Goto (2000) [9] identified gene for cold tolerance in *Drosophila* by using substractive hybridization and reported that the Dca gene, is involved in cold acclimation, and it is upregulated at the transcription level, in *D. melanogaster* flies at 15ºC for 1 day. Similarly, differential gene expression of Dca in *D. subobscura* was found in flies reared at either 13ºC or 22ºCby microarray studies (Laayouni, *et al.*, 2007) [14]. The microarrays and real time-polymerase chain reaction (RT-PCR) techniques have showed that Dca is downregulated after a cold shock at 0ºC for 1–3 h (Sinclair, *et al.*, 2007) [20]. Studies have shown that Dca is most likely involved in cold adaptation and not in response after a cold shock. Arboleda- Bustos, *et al.*, (2011) [1] reported that regucalcin gene is present in all genes of *Drosophila*

Gene expression in diapausing organism is observed in various tissues and brain. The brain is the main regulatory part that serves as the repository of the diapause programme (Giebultowicz and Denlinger, 1986) [8]. Diapause specific proteins are expressed in brain during the pupal diapause inflesh fly, *Sarcophaga crassipalpis* which plays a significant role in silencing many genes, but a few genes are uniquely expressed during diapause (Joplin, *et al.*, 1990) [12]. Many proteins are synthesized in brain of non-diapausing and diapausing fly pupae (Joplin, *et al.*, 1990) [12]. In *S. crassipalpis* the genes are identified prior to expression, during and after diapause in the brain of non-diapausing pupae, and it encodes for heat shock 70 cognate protein, and 28S ribosomal protein (Rinehart *et al.*, 2000) [19], and the cell cycle regulators cyclin E, p21 and p53 (Tammariello and Denlinger, 1998) [22]. Though the cell cycle arrest is presumably common to all diapauses, the stage of the arrest does not appears to be the same in all species (Tammariello, 2001) [21]. The cell of the optic lobe enlarges in the tobacco hornworm *M.sexta* in a G2 arrest during pupal diapause (Champlin and Truman, 1998) [3], similarly, in the cells of *B. mori* during embryonic diapause (Nakagaki, *et al.*, 1991) [16]. Numerous genes are known to regulate the G2/M transition, including two cdc2-related Ser/Thr kinases in *B. mori* (Iwasaki, *et al.*, 1997) [11] and regucalcin gene with unknown regulation (Goto and Denlinger, 2002) [10].

The relation between the *Dca* gene expression and cold stress was studied by Goto (2000) [9]. The Dca gene is up-regulated after acclimation to 15ºC and is single copy in genome, located at position 88D on chromosome 3R, and has some introns with molecular weight of 33.3 KDa (Clowers, *et al.*, 2010) [6]. Nevertheless, in *D. melanogaster*, regucalcin and smp30, gene for thermal adaptation are the names of two distinct genes that code for proteins with 71.9% 5 amino acid identities (Reis, *et al.*, 2011) [18]. The sequence variation in Dca is associated with variation in chill coma recovery time, which indicates its adaptive role in cold tolerance (Clowers, *et al.*, 2010) [6]. Itappears that Dca arose by a duplication event from the ancestral regucalcin like gene after the spilt of *Sophophora* and *Drosophila* subgenera. This gene is present in only a single copy, which bears more sequence to regucalcin than Dca (Arboleda-Bustos and Segarra, 2011) [1]. The function of Dca or regucalcin in insect is not known but in mammals, it regulates regucalcin that is expressed in the cells as a Ca^{2+} binding protein that hold EF-hand motif of the Ca^{2+} binding domain, (Yamaguchi, 2013) [25].

The regucalcin gene is highly conserved in mammals and invertebrates, over 15 species consisting of regucalcin family in vertebrate and invertebrate species have been identified with different function (Yamaguchi, 2011) [24]. In the present study, the regucalcin gene expression in cold acclimated diapause eggs of red cotton bug, *Dysdercus cingulatus* was studied as this gene is reported in various species of *Dorsophila sp.*

Material and Method
The red cotton bug, *Dysdercus cingulatus*, was reared in the laboratory for 2 generation and fed on cotton soaked seeds. After 2 generation of rearing, eggs were taken for experiment. For artificial induction of eggs into diapause, eggs were

acclimated to cold temperature at 5ºC for 24 hours after being freshly laid. Eggs weighing 5 mg were homogenized in 500µl of trizol reagent. Additional 1.0 ml trizol reagent was added to 200 µl homogenized diapause eggs and were gently shaken for 30 seconds, followed with centrifugation at 12,000×g for 10 minutes at 4°C. The mixture on centrifugation results in separate three phases with the upper clear aqueous phase containing the RNA, this phase was transferred to a new tube and to this an equal volume of isopropanol was added and kept at -20°C for 20 minutes and centrifuged at 10,000×g for 10 minutes at 4°C. The pellets were successively washed with 100% and 70% ethanol at 10,000x g for 10 minutes at 4°C and kept for drying. It was then dissolved in 20 µl nuclease free water. Purified RNA was measured with NanoDrop.

The cDNA synthesis was carried out using total RNA as template and reverse transcriptase enzyme. The cycling condition for PCR were initiated at 95ºC for 3 minute, followed by denaturating at 95ºC for 10s, annealing at 55ºC for 10s and extension at 72ºC for 30s.The whole cycle of denaturation, annealing and extension was repeated 40 times, and followed by melting curve analysis (65-95ºC). The primers for amplification of diapause gene were derived from (Vesala *et al.*, 2012) [23] as below (Table 1)

Table 1: The primers for amplification of diapause gene in eggs.

Primer Name	Primer sequence(F) (Forward)	Primer sequence(R) (Reverse)	Amplicon Size (bp)	Primer Annealing Temp. (°C)
Regucalcin	5'-CAGAACAAGACGTACAGGAC-3'	5'-TCATCGCCAATGTAGCGCAT-3'	261	55
COI	5-'GGTCAACAAATCATAAAGATATTGG-3'	5-'TAAACTTCAGGGTGACCAAAAAATCA-3'	708	55

Result & Discussion

The isolated DNA and RNA from the eggs of *D. cingulatus* were seen on agarose gel (Figure 1 and 2). The RNA sample was used for amplification of regucalcin gene with forward and reverse primer mentionedas shown in table (1) and PCR product was checked on the 3% agarose gel.

The regucalcin gene amplication was not observed in RNA and DNA in the eggs of the *D. cingulatus*, as the gene amplification was positive in control sample, shows that the nucleic acids (DNA/RNA) obtained from the eggs does not show regucalcin gene amplification in the PCR product figure (3) and (4).

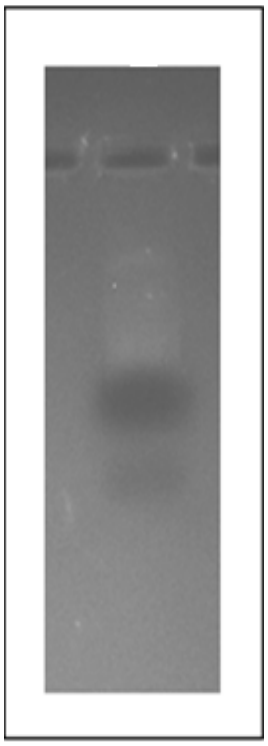

Fig 1: Agarose gel electrophoresis of egg DNA

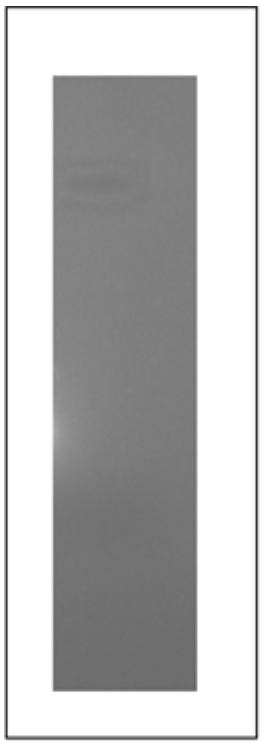

Fig 2: Agarose gel electrophoresis of total egg RNA

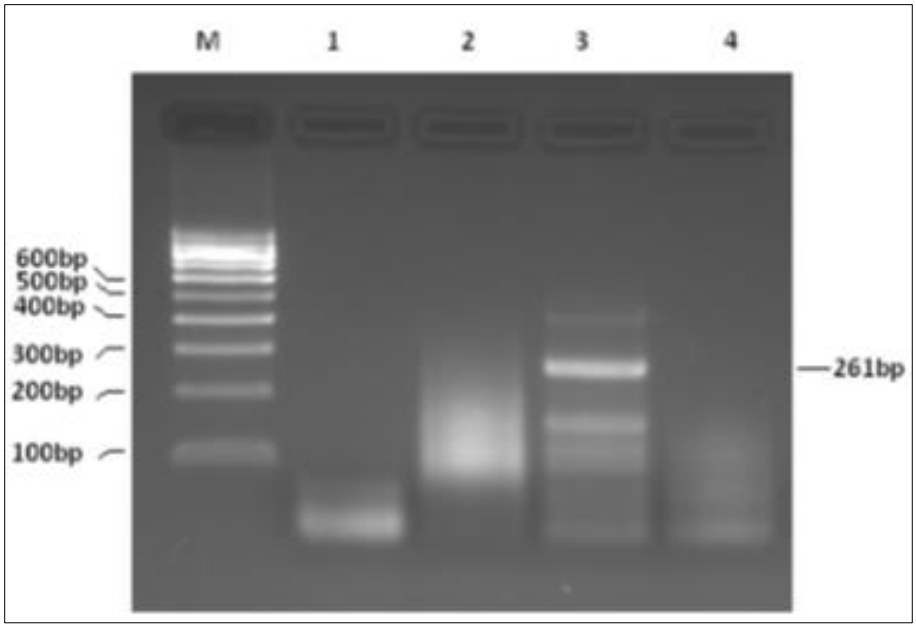

Fig 3: Agarose gel electrophoresis of PCR product of eggs for regucalcin specific primers was performed using 3% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. Each well has each sample DNA loaded. Well No. M ladder 100-1000bp, Well No.1 genomic DNA, Well No2 cDNA, Well No.3 internal positive control DNA, well No.4 Negative Test Control.

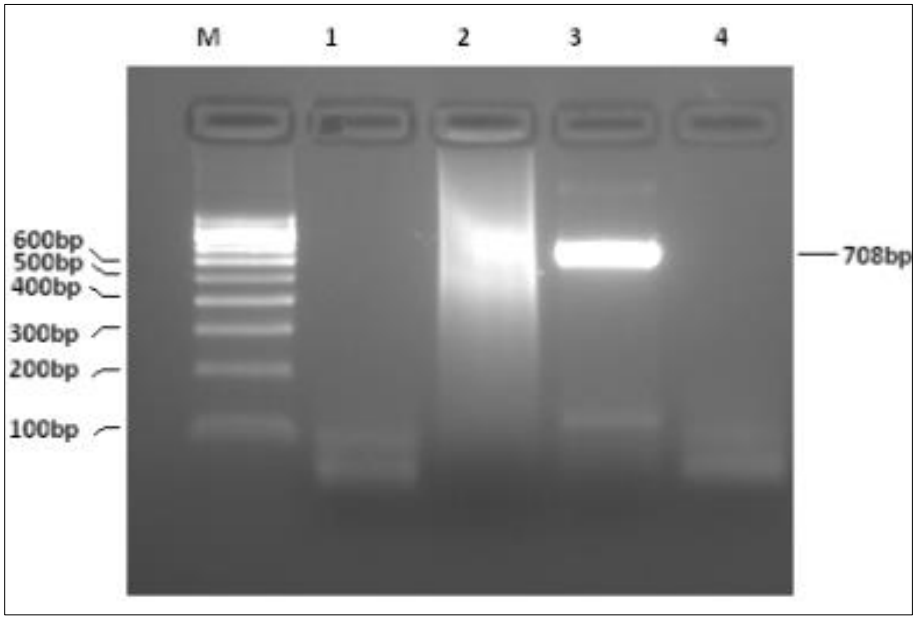

Fig 4: Agarose gel electrophoresis of PCR product of eggs using Internal control gene (COI) PCR product was performed using 3% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. Each well has each sample DNA loaded. Well No. M ladder 100-1000bp,Well No.1 genomic DNA, Well No2 cDNA, Well No.3 internal positive control DNA, well No.4 Negative Test Control.

The percentage hatchability was high at ambient temperature, however when exposed to low temperature result in cold stress induced diapause (Rako and Hoffmann, 2006) [17]. There is no much information available about the effect of cold acclimation diapause and the mechanism behind the process and the gene regulation. The result shows that, the expression of regucalcin in cold acclimated diapausing eggs of *D. cingulatus* is inhibited by cold temperature(Fig 3 and 4). But is upregulated in *D. melanogaster* flies acclimated to 15ºC for 1 day. Differential gene expression of Dca in *D. subobscura* flies reared at either 13ºC or 22ºC was observed (Laayouni *et al.*, 2007) [14] and Sinclair, *et al.*, (2007) [20] By contrast, microarrays and real time-polymerase chain reaction (RT-PCR) techniques showed that Dca is down regulated after a cold shock at 0ºC for 1–3 hrs. In present study we did not detect regucalcin gene at 5°C possibly regucalcin gene may be down regulated at this temperature. Our findings are in consistent with finding of a Sinclair, *et al.*, (2007) [20]. Vesala, *et al.*, (2012) [23] reported that Dca is involved in cold adaptation but not in the response after a cold shock. A similar mechanism may be taking place in the present study. However in *D. montana* regucalcin expressed at a higher level in diapausing than in non-diapausing females maintained in the same day length, but its expression did not differ between short and long day length (Vesala, *et al.*, 2012) [23]. In mammals regucalcin is responsible for calcium binding protein during cold exposure,

with increase in calcium the regucalcin has a suppressive effects on the enhancement of Ca^{2+}dependent and independent protein kinase and phosphatase activities (Yamaguchi, 2013) [25], and the product of regucalcin is also known to play a role in activating the Ca^{2+}pump enzymes and maintaining intracellular calcium homeostasis (Yamaguchi, 2011) [24]. Moreover, regucalcin has been found to regulate gene expression of various proteins related to cell regulation. It is observed that it suppresses cell proliferation. Regucalcin binds to nuclear proteins including calmodulin and DNA, thus, regulate the phosphorylation and dephosphorylation of various proteins that are related to transcription factors. In addition, regucalcin may directly bind on the promoter region of genes, thus, the identification of the base pairs of DNA for binding of regucalcin and responsive elements in the promoter region remains to be determined in relation to its role as a transcription factor of regucalcin. Thus in the present study we conclude that regucalcin may be present however its activation may require certain factors to initiate its action and for this optimum temperature is required. The gene reported in regulating may be the regucalcin gene but their role in low temperature is not studied.

Regucalcin may play an important role in nuclear regulation as reported by (Yamaguchi, 2013) [25]. Of late, the regucalcin gene received much attention due to its role in thermal adaptation in *D. melanogaster* acclimation (Goto, 2000; Clowers *et al.*, 2010) [10, 6]. This cold-adaptive gene is absent in *D. montana*, as well as in other Drosophila subgenus species, where only its ancestral form, regucalcin is present (Arboleda-Bustos and Segarra, 2011) [1]. Based on this studies it reveals that *regucalcin* is not involved in cold acclimation in *D. montana* (II), but it is upregulated under short day conditions inducing reproductive diapause (Kankare, *et al.*, 2010) [13] This gene is not upregulated in diapausing eggs of *D. cingulatus* regardless of the cold acclimation. This, suggests that regucalcin gene may not be important in seasonal adaptation, and is functionally related to diapause as based upon the finding of Arboleda-Bustos and Segarra, (2011) [1]

Thus from the study it shows that regucalcin gene is not expressed in diapause eggs and it may not play any role in cold tolerance diapause eggs.

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