GENE EXPRESSION ANALYSIS OF TRANSCRIPTIONAL CO-ACTIVATORS AND PRO-APOPTOTIC MARKERS IN THE DEVELOPMENT OF *BOMBYX MORI* L. UNDER THERMAL STRESS

A thesis submitted to the University of Calicut

in partial fulfillment of the requirements

For the award of the degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the faculty of sciences

BY

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Under the guidance of

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This is to certify that the thesis entitled "**Gene expression analysis of transcriptional co-activators and pro-apoptotic markers in the development of *Bombyx mori* L. under thermal stress**" submitted to the University of Calicut by Mrs Sajini. K.P. in partial fulfillment for the award of the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by her under my guidance. No part of this work has formed the basis for the award of any degree or diploma previously.

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This is to certify that all corrections /suggestions from the adjudicators have been incorporated in the thesis entitled "**Gene expression analysis of transcriptional co-activators and pro-apoptotic markers in the development of *Bombyx mori* L. under thermal stress**" and the corrected copy is submitted to the University of Calicut for the award of the degree of Doctor of Philosophy in Zoology.

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Gene expression analysis of transcriptional co-activators and pro-apoptotic markers in the development of *Bombyx mori* L. under thermal stress"** is based on the original work done by me under the guidance of Prof. (Dr) C. V. Sreeranjit Kumar and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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DECLARATION

I, hereby declare that the thesis entitled "**Gene expression analysis of transcriptional co-activators and pro-apoptotic markers in the development of *Bombyx mori* L. under thermal stress**" submitted for the degree of doctor of philosophy is the original work carried out by me under the guidance and supervision of Dr. C. V. Sreeranjit Kumar and the dissertation have not formed the basis for the award of any degree, diploma associateship, the fellowship of similar titles. It has not been submitted to any other institution for the award of any degree or diploma.

February, 2026

Sajini. K. P

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The research is dedicated to family

For their everlasting love, affection, Support, and Inspiration.

CONTENTS

ABSTRACT

The silkworm *Bombyx mori* L. is a commercially advantageous insect with significant agricultural value, which exhibits enormous thermal sensitivity. Global warming and climate change pose a focal threat to their growth and reproduction. High temperature negatively impacts nearly all biological processes, including biochemical and physiological processes. While the optimum temperature range for normal silkworm growth is 20ºC to 28ºC and maximum productivity occurs between 23ºC to 28ºC. When temperature exceeds 30ºC that directly harms its health. High temperatures pose a significant threat to all organisms, potentially leading to its death. Exposure to thermal stress compels cells to generate an overabundance of reactive oxygen species and free radicals, which subsequently culminates in oxidative stress. This oxidative burden inflicts significant damage at both the physiological and structural levels within the cell. These ROS known to play an important role in the initiation of apoptosis, a form of programmed cell death, mediated through both mitochondria-dependent and independent pathways. Apoptosis integral to insect development, notably occurring during the metamorphosis of the silkworm. Despite this established role, the specific mechanism by which thermal stress induced oxidative stress and subsequent ROS mediated signaling trigger apoptosis in *Bombyx mori* remains poorly understood. The current study addresses this gap by performing gene expression analysis to investigate the responses of *B. mori* to thermal stress induced apoptosis. Specifically we assessed the differential expression of genes encoding transcriptional co-activators, pro-apoptotic markers and inflammatory response genes across various developmental stages following exposure to thermal stress. This investigation aims to delineate the intricate signaling cascade linking environmental stress, oxidative damage, and programmed cell death in this economically important insect species. For the purpose of evaluating the impact of thermal stress on the gene expression pattern we used the quantitative RT PCR method. Heat stress induced oxidative stress also leads to an imbalance in the antioxidant defense system and it also affects the growth and development of silkworms. In an effort to determine the impact of thermal stress, morphometric parameters of larvae, pupae and adults were studied and to determine the antioxidant activity various biochemical assays were also done. To evaluate the changes in the histological architecture of midgut tissue were also done.

The quantitative and qualitative parameters of cocoons in response to thermal stress were also evaluated.

Following the fourth moult, fifth instar larvae were divided into two groups, control (C) group and thermal stress exposed (T) group. The group 1 was treated with normal rearing temperature about 25±2ºC and the group 2 larvae were treated with 40±2ºC temperature in 1 hr per day during the fifth instar stage. The treatments were also continued during the pupal and adult stage.

In our observations, the high temperature exerts a negative impact on the morphometric parameters of larva, pupa and adult. The length of fifth instar larva, weight of larva, pupa and adult were decreased in the thermal stress exposed group compared to control. The mortality rate was increased in the heat shock exposed group in comparison with control. Moreover there was a significant increase in the lipid peroxidation level in different tissues of different developmental stages of silkworm. This indicates the level of oxidative stress induced by thermal stress. In addition to that there was noted a significant increase and decrease in the antioxidant defense system in the heat shock exposed group of different tissues at different developmental stages of silkworm in contrast to control. Thermal stress exposed groups showed an alternation in the activity of antioxidant enzymes such as SOD, CAT, POX, GST, GPX, and GR.

Furthermore an alteration in the gene expression of BmApaf-1, BmICE-2, Caspase3, BmYki, MBF1, TNFSF5 and IL6 in different developmental stages of silkworm. Thermal stress exerts an upregulated expression of apoptotic related genes such as BmApaf-1, BmICE-2 and Caspase3 gene in different tissues of different developmental stages of silkworm. This indicates the activation of apoptotic pathways in silkworm *Bombyx mori* in response to thermal stress. Upregulated expression of transcriptional co-activator genes (BmYki and MBF1) in the experimental group may protect the organism from thermal stress. Besides that the increased expression of IL6 and TNFSF5 implicated the incidence of inflammation in thermal stress exposed group in comparison with control. Moreover the heat shock protein genes play a critical role in the defense against thermal stress. The upregulated expression of

Hsp70 gene in all the developmental stages indicates the protection against thermal stress.

The present study also analyzed the impact of thermal stress on the various cocoon parameters like length, weight, shell weight, shell ratio, filament length, filament weight and reelability percentage. It is obvious that thermal stress negatively affects the quality and quantity of cocoons produced. So the result revealed that thermal stress induced oxidative stress exerts a negative impact on the overall growth and productivity of cocoons. Moreover the thermal stress induced ROS initiates the activation of apoptotic pathways and also activates the stress response genes to withstand the thermal stress. This study gives an idea about the overall biochemical and molecular aspects of stress response in silkworm on exposure to thermal stress.

Keywords: Thermal stress, antioxidant enzymes, pro-apoptotic genes, transcriptional co-activators, inflammatory response genes.

സംഗ്രഹം

കാർഷികമൂല്യവും താപസംവേദനക്ഷമതയും വളരെ കൂടുതലായ പട്ടുനൂൽപ്പുഴു വാണിജ്യപരമായി പ്രയോജനകരമായ ഒരു പ്രാണിയാണ്. ആഗോളതാപനവും കാലാവസ്ഥാവ്യതിയാനവും ഇവയുടെ വളർച്ചക്കും പ്രത്യുല്പാദനത്തിനും ഒരു ഭീഷണിയായി നിലകൊള്ളുന്നു.ഉയർന്ന താപനില ജൈവതന്മാത്രാപരവും ശാരീരികപ്രവർത്തനപരവുമായ പ്രക്രിയകൾ ഉൾപ്പെടെ മിക്കവാറും എല്ലാ ജൈവ പ്രക്രിയകളെയും പ്രതികൂലമായി ബാധിക്കുന്നു. സാധാരണ പട്ടുനൂൽപുഴുവിന്റെ വളർച്ചയ്ക്ക് ഏറ്റവും അനുയോജ്യമായ താപനില 20°C മുതൽ 28°C വരെയും പരമാവധി ഉല്പാദനക്ഷമത 23°C മുതൽ 28°C വരെയുമാണ്. താപനില 30°C കൂടുന്നത് പട്ടുനൂൽപുഴുവിന്റെ ആരോഗ്യത്തെ നേരിട്ട് ദോഷകരമായി ബാധിക്കുന്നു. താപനിലയിലുള്ള ഉയർച്ച എല്ലാ ജീവജാലങ്ങൾക്കും കാര്യമായ ഭീഷണി ഉയർത്തുന്നു. എന്നാൽ ഉയർന്ന താപനില ചില ജീവജാലങ്ങളുടെ മരണത്തിലേക്ക് തന്നെ നയിച്ചേക്കാം. താപസമ്മർദ്ദത്തിന് വിധേയമാകുന്നത് കോശങ്ങളിൽ അമിതമായി പ്രതിപ്രവർത്തന ഓക്സിജൻ സ്പീഷീസുകളും സ്വതന്ത്ര റാഡിക്കലുകളും സൃഷ്ടിക്കാൻ നിർബന്ധിതരാകുകയും ഇത് പിന്നീട് ഓക്സിഡേറ്റീവ് സമ്മർദ്ദത്തിൽ കലാശിക്കുകയും ചെയ്യുന്നു.ഈ ഓക്സിഡേറ്റീവ് ഭാരം കോശത്തിനുള്ളിലെ ശാരീരികവും ഘടനപരവുമായ തലങ്ങളിൽ ഗണ്യമായ നാശമുണ്ടാക്കുന്നു. മൈറ്റോകോൺട്രിയയെ ആശ്രയിച്ചിട്ടുള്ളതും സ്വതന്ത്രമായതുമായ രണ്ട് പാതകളിലൂടെയാണ് കോശമരണത്തിന്റെ ഒരു രൂപമായ അപോപ്റ്റോസിസ് പ്രധാനമായും സംഭവിക്കുന്നത്.അപോപ്റ്റോസിസ് ആരംഭിക്കുന്നതിൽ റിയാക്റ്റീവ് ഓക്സിജൻ സ്പീഷിസുകൾ നിർണായക പങ്കുവഹിക്കുന്നതായി അറിയപ്പെടുന്നു. പ്രാണികളുടെ വികാസത്തിൽ അവിഭാജ്യ പ്രക്രിയയാണ് അപോപ്റ്റോസിസ്, പ്രത്യേകിച്ച് പട്ടുനൂൽപ്പുഴുവിന്റെ രൂപാന്തരീകരണ സമയത്ത് സംഭവിക്കുന്നത്. ഈ സ്ഥാപിത പങ്കുണ്ടായിരുന്നിട്ടും, ബോംബിക്സ് മോറിയിൽ താപ സമ്മർദ്ദം മൂലമുണ്ടാകുന്ന ഓക്സിഡേറ്റീവ് സമ്മർദ്ദവും തുടർന്നുള്ള റിയാക്ടീവ് ഓക്സിജൻ സ്പീഷീസുകളുടെ മധ്യസ്ഥ സിഗ്നലിങ്ങും അപോപ്റ്റോസിസിന് പ്രേരിപ്പിക്കുന്ന നിർദിഷ്ട സംവിധാനത്തെപ്പറ്റി ഇപ്പോഴും കൃത്യമായ ധാരണയില്ല.താപസമ്മർദ്ദം മൂലമുണ്ടാകുന്ന അപ്പോപ്റ്റോസിസിനോടുള്ള ബോംബിക്സ് മോറിയുടെ പ്രതികരണങ്ങൾ അന്വേഷിക്കുന്നതിനായി ജീൻ എക്സ്പ്രഷൻ വിശകലനം നടത്തി കൊണ്ടാണ് നിലവിലെ പഠനം ഈ വിടവ് പരിഹരിക്കുന്നത്. താപസമ്മർദ്ദത്തിന് വിധേയമായതിനെ തുടർന്ന് വിവിധ വികസന ഘട്ടങ്ങളിലുടനീളം ട്രാൻസ്ക്രിപ്ഷണൽ കോ ആക്ടിവേറ്ററുകൾ, പ്രോ-അപോപ്റ്റോട്ടിക് മാർക്കറുകൾ, കോശജ്വലന പ്രതികരണ ജീനുകൾ എന്നിവ എൻകോഡ് ചെയ്യുന്ന ജീനുകളുടെ ഡിഫറൻഷ്യൽ എക്സ്പ്രഷനുകൾ പ്രത്യേകം വിലയിരുത്തി. സാമ്പത്തികമായി പ്രധാനപ്പെട്ട ഈ പ്രാണിയിനത്തിലെ പാരിസ്ഥിതിക സമ്മർദ്ദം, ഓക്സിഡറ്റീവ് നാശനഷ്ടം ഓക്സിഡേറ്റീവ് നാശനഷ്ടം, പ്രോഗ്രാം ചെയ്ത കോശ മരണം എന്നിവയുമായി ബന്ധിപ്പിക്കുന്ന സങ്കീർണമായ സിഗ്നലിംഗ് കാസ്കേഡ് നിർവചിക്കുക എന്നതാണ് ഈ അന്വേഷണം ലക്ഷ്യമിടുന്നത്.

ജീൻ പ്രകടനമാതൃകകളിൽ താപസമ്മർദ്ദത്തിന്റെ ആഘാതം വിലയിരുത്തുന്നതിന് വേണ്ടി RTPCR സാങ്കേതികവിദ്യ ഉപയോഗിച്ചു. താപ സമ്മർദ്ദം മൂലമുണ്ടാകുന്ന ഓക്സീകരണ സമ്മർദ്ദം ഓക്സീകരണ പ്രതിരോധ സംവിധാനത്തിലെ അസന്തുലിതാവസ്ഥയിലേക്ക് നയിക്കുന്നു. ഇത് പട്ടുനൂൽ പുഴുക്കളുടെ വളർച്ചയെയും വികസനത്തെയും പ്രതികൂലമായി ബാധിക്കുന്നു.

അഞ്ചാമത്തെ വളർച്ചാഘട്ടത്തിലുള്ള ലാർവകളെ നിയന്ത്രിത വിഭാഗം(C), താപ സമ്പർക്ക വിഭാഗം (T) എന്നിങ്ങനെ രണ്ടു വിഭാഗങ്ങളിലായി തരംതിരിച്ചു. നിയ

ന്ത്രിത വിഭാഗത്തിന് സാധാരണ താപനിലയും താപ സമ്പർക്ക വിഭാഗം ലാർവകൾക്ക് താപനിലയും അഞ്ചാം ഘട്ടത്തിൽ പ്രതിദിനം ഒരു മണിക്കൂർ വീതം നൽകി. അതുപോലെ സമാധി, പൂർണ്ണ വളർച്ച , എന്നീ ഘട്ടങ്ങളിലും പരീക്ഷണം തുടർന്നു.

ഞങ്ങളുടെ നിരീക്ഷണങ്ങളിൽ, ഉയർന്ന താപനില പുഴു, സമാധി, പൂർണ്ണവളർച്ചയെത്തിയവ എന്നിവയുടെ ബാഹ്യഘടനാഘടകങ്ങളെ പ്രതികൂലമായി ബാധിക്കുന്നതായി കണ്ടു.

നിയന്ത്രണ വിഭാഗമായി താരതമ്യപ്പെടുത്തുമ്പോൾ താപസമ്മർദ്ദത്തിന് വിധേയമായ അഞ്ചാംഘട്ട പുഴുക്കളുടെ നീളവും, പുഴു, സമാധി, മുതിർന്നവ എന്നീ വളർച്ച ഘട്ടങ്ങളിലെ ഭാരവും കുറഞ്ഞുവരുന്നതായി കണ്ടു. നിയന്ത്രണ വിഭാഗവുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ താപസമ്മർദ്ദത്തിന് വിധേയമായ വിഭാഗത്തിലെ മരണനിരക്കും വർദ്ധിച്ചു. താപസമ്മർദ്ദത്തിന് വിധേയരായ വിഭാഗങ്ങൾ SOD CAT POX GST GPX GR തുടങ്ങിയ ആന്റിഓക്സിഡന്റ് എൻസൈമുകളുടെ പ്രവർത്തനങ്ങളിൽ വ്യതിയാനം കാണിച്ചു.

പട്ടുനൂൽപ്പുഴുവിന്റെ വിവിധ വളർച്ച ഘട്ടങ്ങളിലെ വിവിധ കോശങ്ങളിലെ കൊഴുപ്പിന്റെ പെറോക്സീകരണ അളവിൽ ഗണ്യമായ വർദ്ധനവുണ്ടായി. താപ സമ്മർദ്ദം മൂലം ഉണ്ടാകുന്ന ഓക്സീകരണ സമ്മർദ്ദത്തിന്റെ അളവ് ഇത് സൂചിപ്പിക്കുന്നു. അതിനുപുറമെ നിയന്ത്രിത വിഭാഗത്തിൽ നിന്ന് വ്യത്യസ്തമായി പട്ടുനൂൽ പുഴുവിന്റെ വിവിധ വളർച്ചാഘട്ടങ്ങളിലെ വിവിധ കോശകലകളുടെ പരീക്ഷണ വിഭാഗങ്ങളിലെ ഓക്സീകരണ പ്രതിരോധ സംവിധാനത്തിൽ ഗണ്യമായ വ്യതിയാനം രേഖപ്പെടുത്തിയിട്ടുണ്ട്. കൂടാതെ പട്ടുനൂൽപ്പുഴുവിന്റെ വിവിധ വികസന ഘട്ടങ്ങളിൽ BmApaf-1,BmICE -2, caspase 3, BmYki,MBF1,IL6,TNFSF5 എന്നീ ജീനുകളുടെ ആവിഷ്കാരം പരീക്ഷണ വിഭാഗങ്ങളിൽ വ്യത്യാസപ്പെടുന്നതായി കാണുന്നു. പട്ടുനൂൽപ്പുഴുവിന്റെ വിവിധ വളർച്ചാഘട്ടങ്ങളിലെ വിവിധ കോശങ്ങളിൽ താപ സമ്മർദ്ദം കോശ നശീകരണവുമായി ബന്ധപ്പെട്ട BmApaf-1,BmICE -2, caspase 3 ജീനുകളുടെ പ്രകടനം വർദ്ധിക്കുന്നതായി കാണപ്പെട്ടു. താപസമ്മർദ്ദത്തിന് മറുപടിയായി പട്ടുനൂൽപ്പുഴുവിലെ കോശ നശീകരണ പ്രവർത്തനങ്ങൾ സജീവമാക്കുന്നതിനെ സൂചിപ്പിക്കുന്നു. പരീക്ഷണ വിഭാഗങ്ങളിൽ അനുരൂപണ ഉദ്ദീപന സഹായി ജീനുകളുടെ(BmYki,MBF1) വർദ്ധിച്ച പ്രകടനം ശരീരത്തെ താപസമ്മർദ്ദത്തിൽനിന്നും സംരക്ഷിച്ചേക്കാം. കൂടാതെ IL6,TNFSF5 എന്നിവയുടെ വർദ്ധിച്ച പ്രകടനം പരീക്ഷണ വിഭാഗത്തിൽ ക്ഷോഭീകരണം സംഭവിക്കുന്നതിന് സൂചിപ്പിക്കുന്നു. കൂടാതെ താപ സമ്മർദ്ദത്തിനെതിരായ പ്രതിരോധത്തിൽ താപ സമ്മർദ്ദ മാംസ്യജീനുകൾ നിർണായക പങ്കുവഹിക്കുന്നു. എല്ലാ വികസന ഘട്ടങ്ങളിലും HSP 70 ജീനിന്റെ വർദ്ധിച്ച പ്രകടനം താപസമ്മർദ്ദത്തിനെതിരായ സംരക്ഷണത്തെ സൂചിപ്പിക്കുന്നു. കൊക്കൂണുകളുടെ പാരാമീറ്ററുകളിൽ താപസമ്മർദ്ദത്തിന്റെ സ്വാധീനം നിരീക്ഷിച്ചപ്പോൾ ഉല്പാദിപ്പിക്കുന്ന കൊക്കൂണുകളുടെ ഗുണനിലവാരത്തെയും അളവിനെയും താപസമ്മർദ്ദം പ്രതികൂലമായി ബാധിക്കുന്നുവെന്നത് വ്യക്തമായി. കൂടാതെ താപസമ്മർദ്ദം മൂലമുണ്ടാകുന്ന ROS കോശ നശീകരണ പാതകളുടെ സജീവമാക്കൽ ആരംഭിക്കുകയും താപസമ്മർദ്ദത്തെ നേരിടാൻ സമ്മർദ്ദ പ്രതികരണ ജീനുകളെ സജീവമാക്കുകയും ചെയ്യുന്നു. ഈ പഠനം താപസമ്മർദ്ദത്തിന് വിധേയമാകുമ്പോൾ പട്ടുനൂൽപ്പുഴുവിന്റെ സമ്മർദ്ദ പ്രതികരണത്തിന്റെ മൊത്തത്തിലുള്ള ജൈവരാസിയവും, തന്മാത്രപരവുമായ വശങ്ങളെക്കുറിച്ച് ഒരു വിപുലമായ വിശദീകരണം നൽകുന്നു .

LIST OF ABBREVIATIONS

B. mori	-*Bombyx mori*
CAT	- Catalase
GPx	- Glutathione peroxidase
GR	-Glutathione reductase
GST	- Glutathione S– transferase
H_2O_2	-Hydrogen Peroxide
Hsp	-Heat Shock Protein
LPO	-Lipid Peroxidation
ROS	- Reactive Oxygen Species
SOD	-Superoxide dismutase
GSSG	- Oxidized Glutathione
POX	- Peroxidase
BmYki	-*Bombyx mori* Yorkie
MBFI	-Multiprotein bridging factor1
BmApaf1	-*Bombyx mori* Apoptotic protease activating factor 1
BmICE 2	-*Bombyx mori* Intertleukin1β converting enzyme 2
TNFSF5	-Tumor necrosis Factor Super Family 5
IL6	- Interleukin 6

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INTRODUCTION

1. INTRODUCTION

1.1. Sericulture–an outline

Sericulture, artwork, and the scientific execution of rearing silkworms for making silk is a labor-intensive; farm-based economically, and commercially valuable activity. It is an excellent, less time-consuming (40 days) approach to assuage poverty in rural areas and this practice does not require higher education (Goswami and Bhattacharya, 2013; Mubin, 2013; Rahmatulla, 2012). Silk was invented in China in the period between 2600 and 2700 BC. Silk possessing multiple properties like tensile strength, smoothness, elegance, durability, and captivating shine, has rightfully earned the title of queen of textiles. Beyond these well-known attributes, silk exhibits other impressive properties, including the ability to resist heat, excellent water absorption capacity, inherent luster, and exceptional dyeing competence (Rahmathulla, 2012). Globally above 95 % of the total raw silk production is accomplished by Asia, which is the world's leading producer. While China and India are the world's largest producers of silk, Uzbekistan, Vietnam Thailand, Brazil, and South Korea are also significant contributors (Anonymous, 2021).

1.2. India's Sericulture Scenario

The silkworm, a Lepidopteran insect, is specifically cultivated for the production of silk and constitutes the back bone of the sericulture industry (Ashraf and Qamar, 2023). Currently, India holds the position of the world's second largest producer of silk, achieving an annual output of 36582 MT. India serves as a major natural habitat for a wide range of silk moths; moreover, it possesses a shelter for a wide variety of silk-generating fauna. This diversity has allowed India to succeed in the inimitable position of being the only country in the world to produce all four varieties of natural silk, specifically Mulberry, Tasar, Muga, and Eri. Among these, Muga silk with its golden yellow tinge is a unique dominance of India. Most silk generated in India is mulberry silk, which is turned out by the silkworm *Bombyx mori*. From 2022 through 2023, India's sericulture industry yielded 75.60 % (27,654 MT) Mulberry silk, 3.60% (1318 MT) Tasar silk, 20.09 % (7349 MT) Eri silk, and 0.712% (261 MT) Muga silk from the overall raw silk output (Anonymous, 2022). In India, the sericulture field provides employment to about 9.43 million people (Anonymous, 2019-2020).

Sericulture is established particularly in the Indian states of Kerala, Tamil Nadu, Karnataka, West Bengal, Andhra Pradesh, Gujarat, Maharashtra, Jammu and Kashmir, Orissa, Rajasthan, Bihar, and Uttar Pradesh (Gurjar *et al*., 2018). In India sericulture plays a vital role in the national economy, women's upliftment, employment generation to the society, and rural development. The sericulture practices sustain lively hoods for more than six million families across 59,000 villages in the country and the silk section is one of the major foreign trade earners for the country. Approximately 53% of women in rural areas are involved in sericulture activities like rearing and reeling which offers sufficient income- generating opportunities. Sericulture relatively requires 'low capital investment', for this reason, sericulture can be accessible to both small and large-scale landholders too. So sericulture practice is convenient for farmers with varying land sizes. Sericulture plays a critical role in supporting rural livelihoods, as evidenced by the fact that primary producers in rural communities retain nearly 57% of the final value of silk fabrics, even though consumption is primarily concentrated among the prosperous urban segments. This economic linkage underscores sericulture's substantial contribution to rural income (Gangopadhyay, 2008). The central aspirations of the Indian government are to produce good-quality silk. The Central Silk Board in association with other National level R&D Institutions, Universities, Central and State level Sericultural Departments, and other Private Research Institutes collaborates and works together to improve the quality of silk.

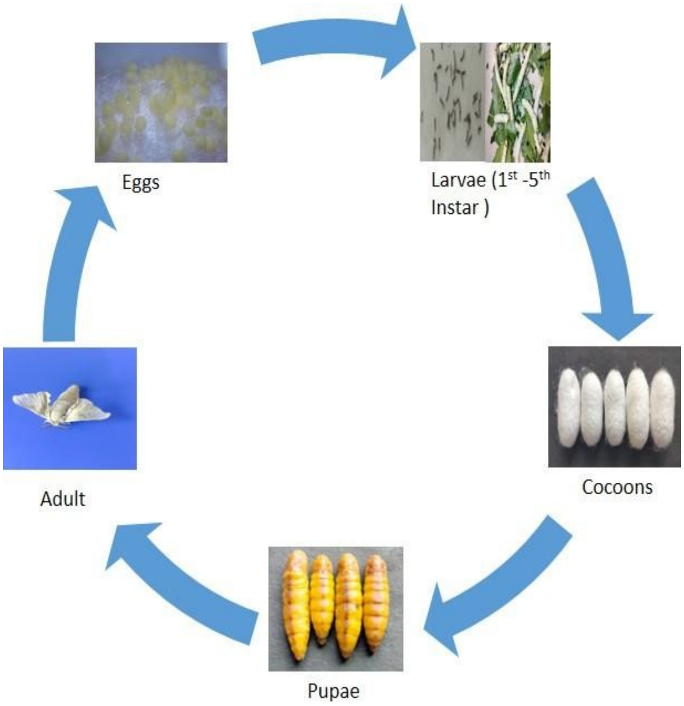
1.3. Silkworm as a biological model organism

Silkworm *Bombyx mori* is a monophagous, economically important insect extensively used in biological experiments as a model organism. It has plenty of reasons behind this being used as a study organism, especially 'low breeding cost, short generation time, large progeny size and apparent genetic background'. Moreover, completion of the genome and the similarity of some of the silkworm genes with human genes, related to hereditary diseases are important credits for selecting silkworms as a representative organism for learning human disease (Meng *et al*., 2017). In 2003 China and Japan initiated the task to study the entire genetic code of silkworms by conducting a 'silkworm genome project' and this study resulted in the 'draft map, fine map, and multi strain genome re-sequencing of the silkworm'. This experiment greatly influenced the growth of ‘sericulture science’ (Xia *et al*., 2004; Xia *et al*., 2009). Silkworms possess 28 pairs of chromosomes greater than that of *Drosophila melanogaster* (4 pairs), and the silkworm genome contains 450 million bp, which is four times greater than that of Drosophila and 1/6 of the human genome (Meng *et al.,* 2017). The death of a silkworm, regardless of whether it is caused by genetic factors or external forces, does not raise any specific bioethical concerns (Chen *et al*., 2014). Silkworms have moderate body size and are easy to handle in the laboratory, especially for dissecting and collecting internal tissues and organs. Moreover, like mammals, silkworms are used in ‘oral administration and intravenous injection experiments’. Nowadays silkworms have been used in varying research areas like ‘antitumor studies, human diseases, environmental safety monitoring and testing of antimicrobial agents (Meng *et al*., 2017) etc.

1.4. Silkworm developmental stages

The development of silkworm *Bombyx mori* encompasses four different life stages; they are egg, larva, pupa, and adult. Depending on the ‘ethnic characteristics and climatic conditions’ the period of life cycle extends between six to eight weeks (45-55 days). Eggs are small and oval-shaped with yellowish color pigmentation; generally, this stage lasts 9-10 days. The larval stage is the feeding stage of silkworm consisting of five instars and four moults. The larval stage exists between 24-28 days. The length of the newly hatched larva is about 4-6 mm and the full- grown fifth instar larva is about 6-8 cm. The matured fifth instar larvae stopped feeding and started spinning by the secretion of a clear sticky fluid from the salivary glands through the spinneret, which turns into fine silk. The complete spinning and cocoon formation takes place within 3-4 days. Then the larva turns into pupa (Chrysalis) within 8-10 day time duration. The pupa is conical-shaped and brownish in colour. The pupa undergoes complete metamorphosis, forming adult structures such as ‘antennae, wings, and copulatory apparatus’. After finishing the cocoon stage the fully developed adult emerges through the opening and undergoes mating; the female moths lay around 300-400 eggs and die immediately. Compared to male moths, female moths are larger in body size with smaller antennae and smaller wings. The male moth consists of large antennae and large wings with smaller body size. The adult life lasts for 3-4 days (Krishnaswamy *et al*., 1973; Vasantharaj& Ramamurthy, 2015).

Figure 1.1. Life cycle of Silkworm



1.5. Types of silkworm

India is the most diverse silk fauna existing country in the world, the four types of silkworm varieties are Mulberry, Muga, Tasar, and Eri. These four silkworms vary based on their feed in the larval stage. Mulberry silkworm *Bombyx mori* is traced back to China. Conversely all other silkworms such as the Muga silkworm (*Antheraea assama*), Eri silkworm (*Philosamiaricini*), and Tasar silkworm (*Antheraea mylitta*) are recognized as originating from India. Mulberry leaves are the only food resource behind the production of mulberry silk. Muga silkworms feed on the fragrant leaves of Som and Soalu plants and Eri silkworm larvae feed on castor leaves. The Tasar silkworm needs specific host vegetation, thriving predominantly on the leaves of Asan and Arjun trees. The Oak Tasar silkworm Antheraea *proylei* consumes leaves of the natural food plant oak (Chand *et al*., 2023).

There are three commercially important silkworm races, classified based on their number of generations per annum. They are referred to as univoltine, bivoltine and multivoltine. Univoltine silkworms produce one generation per year. Mean while, the bivoltine silkworms complete two generations per year, however, multivoltine silkworms produce more than two crops per year.

1.6. Effect of thermal stress on silkworm

Extreme weather events including forest fires, heavy precipitation, and raising temperatures are becoming more often and intense due to climate change and global warming (Frame *et al*., 2020). The impact of climate change is evidenced by a decline in some insect populations, especially the moths (Dar and Jamal, 2021b). Global warming poses a significant scare to insect populations by impacting their survival and reproductive success as a result of elevated temperatures. This fact is a key contributor to the fade in sericulture production (Abdel Hady *et al*., 2021; Gonzalez-Tokman *et al*., 2020; Stazione *et al*., 2019). Insect's growth, development, reproduction, and distribution are remarkably affected by varying temperatures (Cossins and Bowler, 1987; Worner, 1988; Bale *et al*., 2002). Silkworms are thermally sensitive and economically important insects, rearing at agreeable temperatures between 25-28ºC. Temperature, humidity, and photoperiod fluctuations are all important factors in the growth, "development and production of high-quality cocoons" (Hussain *et al.*, 2011). The incessant domestication is the most important reason for the sensitivity of silkworms against environmental vacillations and leads to the inability to survive naturally (Hussain *et al*., 2011). Seasonal variations in the environmental components immensely affect the genotypic expression which results in reducing the phenotypic output in the form of cocoon characteristics such as cocoon weight, shell weight, and cocoon shell ratio (Rahmathulla, 2012). The normal development of silkworms occurred in the optimum temperature ranges of 20ºC to 28ºC and the greatest yield of cocoons ranges from 23ºC to 28ºC. Temperature beyond 30ºC promptly affects the larval health. In early instars, if the temperature goes below 20ºC it will slow down all the physiological processes (Rahmathulla, 2012). In the summer seasons the temperature attain 30ºC or higher in tropical regions like southern China, India, and Thailand which affects the growth of silkworms (Hossein *et al*., 2008). In day time the temperature leaps up in India. In summer seasons it may leap up to a range of 35-40ºC or even more. This inconsistency in temperature exerts a deleterious effect on the pupation and survival of silkworms, specifically for bivoltine strains. This temperature fluctuation ultimately leads to the loss of the sericulture industry. Like all organisms, in silkworms, most of the characteristics are controlled by genes, but temperature has a special influence on controlling some specific characteristics of silkworms (Watanabe, 1918, 1919, 1924, 1928; Kogure, 1933).

Elevated temperature not only affects growth and development, it affects nearly all biological processes including physiological as well as biochemical reactions (Hazel, 1995; Willmer *et al*., 2004), and finally has an effect on the quality or quantity of cocoon crops. Silkworm larvae and pupae were exposed to varying thermal stress, 31ºC and 36ºC, resulting in reduced haemolymph concentration, and the order of reduction was more at 36ºC than 31ºC (Malik and Malik, 2009).

Climatic factors and the physiological status of the parental strains are two important factors affecting the reproductive success of the silkworm. Diverse temperature regimes exert a significant influence on key silkworm characteristics, specifically affecting cocoon weight and reproductive capacity. Ultimately, the quality of the cocoon, the traits of the adult moths, and the reproductive success of the strains determine their overall commercial viability (Sing and Samson, 1999). Male adults become sterile when exposed to high temperatures (Sugai and Hanaoka, 1972). The egg-laying capacity of *Bombyx mori* has been influenced by the rearing environmental thermal level and silkworm genotype. During the fostering of silkworms, adverse environmental conditions lead to the poor performance of parental lines (Kumar and Singh, 2012). Ayuzawa *et al*., (1972) reported the diminished egg recovery and increased number of unfertilized egg production under high temperature and low relative humidity. Decreased production of quality eggs and increased production of unfertilized eggs under adverse temperature conditions were also reported by Hussain *et al*., (2011) and Kumar and Singh, (2012). The hatchability of Nistari breeds declined drastically to zero when exposed to 43ºC temperature stress (Sinha and Sanyal, 2013). When kept below 30ºC, the *Bombyx mori* cross breed (PM X CSR2) inhibit oviposition, while exposure to below 40ºC cause larval mortality (Wanule and Balkhande, 2013). Akin to the larval stage, high temperature also wields an effect on the embryonic stage, resulting in a reduction in the hatchability of eggs (Taha, 2013). Reduced fecundity in the CSR18 *Bombyx mori* breed when subjected to temperature stress above 42ºC. Temperature exceeding 42ºC affects the performance of the female reproductive system (Paul and Keshan, 2016). Silkworm survival, cocoon, and pupal yield were decreased during thermal stress (Li *et al*., 2018; Mir and Qamar, 2018).

Five selected breeds of silkworm *Bombyx mori* exposed to two different high temperatures revealed a deleterious effect on nine quantitative characters, such as larval weight, cocoon weight, filament weight, shell weight, shell ratio, filament length, denier, renditta, and effective rate of rearing (ERR %). The selected breeds CSR2, CSR4, KA, JROP, and NB4D2 experienced a poor performance, marked by a significant reduction in larval weight, filament length, cocoon weight, and ERR (Nabizadeh and Kumar, 2010). The biological trait larval mortality and effective rate of rearing are adversely affected under varying thermal stress in strains of silkworm *Bombyx mori* (Chavadi *et al*., 2006). Beyond impacting the silkworm's growth, development, and economic traits, temperature also has a negative effect on it's essential gut flora. The abundance of gut flora severely decreased with increasing temperature (Sun *et al*., 2017).

High temperatures (45ºC) have the potential to exert an effect on both young and late larval instars. Male and female larvae of varying instars were exposed to different temperatures; as a result, complete larval mortality occurred in all larval instars, irrespective of sex and strains. According to varying thermal stress, all larval instars showed alternation in the expression of heat shock protein genes (Punyavathi and Manjunatha, 2020). Low temperature exerts a deleterious effect on the growth and development of silkworms even though high temperature affects the immune system as a result silkworms are susceptible to different types of diseases (Li *et al* ., 2012b).

1.7. Oxidative stress and apoptosis

The elevated temperature has a direct impact on "cell organizations", metabolic processes (Walter *et al*., 1990), and "physiological activities" of silkworms (Jia *et al*., 2011). Generally, thermal stress responses are related to the augmented production of reactive oxygen species generating oxidative damage (Zhang *et al.*, 2014). Normally the endogenous oxidative metabolism as well as from different exogenous sources results in the formation of ROS, which consists of partly reduced metabolites of oxygen such as superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals(OH^-) (Livingstone, 2001 ;Thannickal and Fanburg, 2000).

Although ROS does have a range of functions, for example, an ideal level of intracellular ROS needed for the mechanism of cellular homeostasis (Sena and

Chandel, 2012), whereas a slight amount of ROS rendered as a signaling molecule in the survival pathways (Martindale and Holbrook, 2002). Excessive production of reactive oxygen species (ROS) can cause oxidative stress (Selvakumar *et al*., 2013). The status of oxidative stress is defined as the disparity between the formation of ROS and the regulation of the antioxidant defense system (Lopez-Martinez *et al*., 2008). Formation of ROS leads to the increased generation of lipid peroxidation, causes DNA damage, and oxidation of protein (Nordberg and Arner, 2001), and eventually leads to membrane damage and lethargy of enzymes (Livingstone, 2001).

Antioxidant enzymes and non-enzymatic antioxidants are used to counteract the generation of high amounts of ROS (Felton and Summers, 1995). Like any other organisms, silkworms also possess a defensive antioxidant system (Micheal and Subramanyam, 2013). Enzymatic antioxidants primarily consists of Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S- transferase (GST) (Halliwell & Gutteridge, 2001). Conversely key examples of non-enzymatic antioxidants are ascorbic acid, vitamin E, and reduced glutathione (GSH) (Felton and Summers, 1995). Each enzyme plays it's role in combating oxidative stress. Superoxide anion can be converted to molecular oxygen and hydrogen peroxide by SOD (Halliwell & Gutteridge, 2001), although the catalytic enzyme CAT (Halliwell & Gutteridge, 2001; Switala & Loewen, 2002) and POX (Meng *et al*., 2009) convert hydrogen peroxide to molecular oxygen and water. GPX engaged in the detoxification of H_2O_2 as well as reduction of organic hydroperoxides and lipid peroxides, these actions are facilitated by reduced glutathione (GSH,) which functions as a hydrogen donor. The enzyme glutathione reductase is liable for the reversion of reduced glutathione (GSH) from oxidized glutathione (GSSG) (Halliwell & Gutteridge, 2001). The hydro peroxides formed by the byproduct of lipid peroxidation, can be removed by the enzyme GST. The activity of antioxidants may not be enough to control the excessive ROS produced and it will lead to oxidative stress (Sen and Packer, 1996).

Thermal stress exerts oxidative damage to the body of bivoltine (SK6 and SK7) and polyvoltine (Nistari and Sarupat) silkworm strains when exposed to 35ºC and 40ºC. High temperatures caused an increase in hydrogen peroxide concentration in both bivoltine and polyvoltine strains showed a significant increase in MDA concentration, which indicates a higher level of lipid peroxidation in their cells due to the thermal stress. To counter the oxidative damage induced by high temperatures, antioxidant enzymes like SOD, CAT, and POX were increased (Magana *et al*., 2021). Silkworms exposed to acute thermal stress displayed a significant decline in fat body catalase activity (Nabizadeh and Kumar, 2010). Stress causes cells to undergo apoptosis or necrosis as a result of structural damage to key components like lipids, proteins and nucleic acids (Sen and Packer, 1996). These cellular injuries and the intensity of lipid peroxidation determine the depth of oxidative stress (Birben *et al.,* 2012). Analogous to numerous other cellular stresses, heat stress possesses the ability to induce apoptosis (Chowdhury *et al*., 2006). Unwanted and potentially perilous cells are being eliminated by the physiological process called apoptosis (Smith *et al*., 1989). The term apoptosis is coined by Kerr, Wyllie, and Currie, derived from a Greek word**,** meaning 'dropping off or falling off petals from flowers' (Nossing and Ryan 2023).

Apoptosis plays different roles such as tissue homeostasis (White, 1996), regulation of development and organ differentiation process, and confrontation against environmental stress (Fulda & Vucic, 2012). Apoptosis is also linked with many human diseases (Singh *et al*., 2019). It has two important pathways, intrinsic and extrinsic (Czerski & Nuñez, 2004). ROS shows important roles in extrinsic (Circu & Aw, 2010) as well as intrinsic pathways (Ryter *et al*., 2007). The intrinsic pathway also known as the mitochondrial apoptotic pathway (Qin *et al*., 1999) is triggered by intracellular signals and extrinsic pathway activated by the transmembrane receptor-mediated interactions (Elmore, 2007). In the intrinsic pathway, mitochondria release cytochrome C making an apoptosome complex with Apaf-1 and ATP, which activates caspase-9. The activated caspase-9 activates caspase-3 and commences the apoptotic process (Qin *et al*., 1999). In the extrinsic pathway, plasma membranes carry cell death receptors that have been activated by the binding of extracellular ligands (Jin & El-Deiry, 2005) and finally activate the effector caspases (Grütter, 2000). Normally insect larval-specific tissues are detached during the programmed cell death mechanism (Li *et al*., 2010). In silkworms, the silk gland is the silk-producing larval specific organ(Hu *et al*., 2018), which can be decadent quickly following the spinning stage (Li *et al*., 2010).

Caspases are a major family of evolutionarily conserved cysteine-dependent aspartate –specific proteases that play a central role in apoptosis (Wang and Gu, 2001; Koonin and Aravind, 2002) which have strong cleavage site specificity towards a four amino acid motif ending with an aspartate residue (Talanian *et al*., 1997)**.** The caspases consist of two main branches such as pro-inflammatory ICE-like subfamily and apoptotic caspase subfamily (Wang and Gu, 2001). In cells, generally, all caspases present as an inactive form of proenzymes. They normally show a canonical structure that consists of three different regions such as N terminal pro-domains, a large catalytic domain, and a small catalytic domain (Talanain *et al*., 1997). Based on the length of the pro-domain, caspases can be divided into two groups such as initiators (long pro-domains) and effectors (short pro-domains). Again initiators are divided into two groups based on their N-terminal prodomain, they are CARD (caspase recruitment domain) and DED (death effector domain) (Talanian *et al*., 1997; Chen *et al*., 1998).

Silkworm *Bombyx mori* comprises five caspase family homologs with two initiators and three effectors by cloning from silkworm BmE cells. The initiators such as *BmDredd*, *BmDronc* and effectors are *BmCaspase*-1, *BmICE,* and *BmCaspase–N* (Zhang *et al*., 2010). In *Bombyx mori*, the first informed effector caspase is *Bm Caspase*-1, which is 1291 bp long, it codes for 293aa with one exon at chromosome 10 (Pei *et al*., 2002).

ROS is one of the major inducers of apoptosis (Chen *et al*., 2015). The thermal stress brings about the production of ROS. The thermal stress-induced apoptotic study has not yet been reported in silkworms. The apoptotic-related genes are identified by Zhang *et al*., (2010). The oxidative stress-induced apoptosis is not well understood in insects. For filling this gap, Chen *et al*., (2015) studied the impact of hydrogen peroxide-induced oxidative stress on apoptosis of silkworm ovary BmN- SWU1 cells. H_2O_2 induction results in the production of ROS which leads to oxidative stress and eventually the cells enter into the mitochondrial apoptotic pathway. Survival silkworm *Bombyx mori* under azaserine-induced oxidative stress, investigated by Muthangi, (2020). They studied the antioxidant enzyme activity along with the expression analysis of apoptotic and antiapoptotic genes.

Some broad-spectrum insecticides used in agriculture for controlling Lepidopteran pests lead to the production of oxidative stress and significantly affect the survival of *Bombyx mori.* The phoxim negatively impacts the survival of silkworm (*Bombyx mori*) when transported by air and water currents. Phoxim induces apoptosis in columnar cells and goblet cells of the silkworm *Bombyx mori*. In the case of *Bombyx mori*, the phoxim also affects food consumption, growth, reproduction, and oxidative stress. Use of this type of organophosphate (Gu *et al*., 2014), environmental stresses such as ultraviolet light, heat shock, and oxidative stress lead to tremendous loss to the sericulture industry.

1.8. Heat shock protein-HSP 70

When insects faced various types of stresses like thermal stress, chemical stress, and physical stresses, they rapidly responded by producing a novel set of polypeptides known as heat shock proteins(HSPs) (Zhao and Jones, 2012). The initial portrayal of cellular stress response was presented by Ritossa, (1962, 1963) in *Drosophila melanogaster.* The discovery of "heat shock protein" was reported by Tissieres *et al*., (1974) under rapid increase in temperature. Based on the molecular weight and function, HSPs are classified as small HSPs (20-40KD), HSP60, HSP70, HSP90, HSP110 (Parsell and Lindquist, 1993), HSP40, HSP100 and HSP10 (Zhao and Jones, 2012). When facing elevated temperatures in the environment, HSP production is a vital survival mechanism employed by various organisms (Parsell and Lindquist, 1993). The essential role of HSPs is environmental adaptation, both evolutionary and ecological and they also play a crucial role in different biological processes like embryogenesis, (Cobrerose *et al*., 2008) morphogenesis (Gunter and Degnan, 2007), and diapause (Rinehart *et al*., 2007). HSPs also help in protein folding or avoiding improper folding of proteins and have a specific role in protein repair mechanisms (Augustyniak *et al*., 2009, Xu *et al*., 2011).

In eukaryotes, HSP 70's are located in four major cellular compartments, cytoplasm, mitochondria, endoplasmic reticulum, and chloroplast (Renner and Waters, 2007). Among the families of HSPs, the cytoplasmic HSP70 exhibits the highest degree of conservation and has been comprehensively investigated in the circumstance of heat shock (Krebs and Feder, 1997; Bahrndorff *et al*., 2009; Calabria *et al*., 2012). It is the most abundant HSP family with the highest level of transcription but it is susceptible to different harmful stimuli (Shu *et al*., 2011). HSP70 family proteins contain three distinct domains; around 400 amino acids comprising an N-terminal adenosine triphosphate (ATPase) domain, a substrate binding domain with about 200 amino acids, and a highly variable C-terminal domain (Renner and Waters, 2007). Cytoplasmic HSP70 family further classified into two, heat and other environmental stress-inducible HSP70, normally expressed in very low amounts and the other one is cognate heat shock 70 proteins (HSC 70) which are constitutively expressed under normal conditions and faintly increased expression against thermal stress (Kiang and Tsokos, 1998; Mahalingam *et al*., 2005; Daugaard *et al.*, 2007). HSPs have a specific role in the control mechanism of programmed cell death processes. HSP 70 repressed the apoptosis process by inhibiting caspase activity (Mosser *et al*., 1997).

1.9. Transcriptional co-activators

1.9.1. BmYki- *Bombyx mori* Yorkie

Yorkie (Yki) is a transcriptional co-activator present in *Drosophila melanogaster* and is a key downstream effector of the hippo signaling pathway which regulates organ development via cell proliferation and apoptosis. BmYki is a Yorkie (Yki) ortholog; it has a 1314 bp length coding sequence containing a protein of 437 amino acids with two WW domains (Zeng *et al*., 2017). BmYki has alternative isoforms that perform different transcriptional co-activator activity. BmYki1, BmYki2, and BmYki3 are alternative isoforms in which BmYki3 encodes 445 amino acid residues that promote cell migration and division and increase the size of cultured cells and wing discs. BmYki could improve cell migration, cell cycle, and cell size; however, the effect of BmYki1 and BmYki2 on cell proliferation was lesser than that of BmYki3 (Liang *et al*., 2019).

1.9.2. MBF1-Multiprotein bridging factor1

Transcriptional co-activator MBF1 was first isolated from the posterior silk gland of silkworm *Bombyx mori (*Li *et al*., 1994). MBF1 is usually accountable for linking the transcriptional factors with gene-specific regulators. Multiprotein bridging factor-1(MBF1), preserved among eukaryotes**,** (Takemaru *et al*., 1997) discovered in adult moth of silkworm required for *in vitro* transcriptional activation by the nuclear receptor Fushi Tarazu Transcriptional Factor 1 (FTZ-F1). By linking a nuclear hormone receptor family, transcription factor BmFTZ-F1, and TATA box binding protein (TBP), BmMBF1 recruits the general factor TBP to a promoter carrying the BmFTZ-F1 binding site, which initiates the transcription of RNA polymerase II (Li *et al*., 1994).

MBF1 plays a fundamental role in gene expression. In animal cells, the location of MBF1 changes within the cell from one organelle to another; it facilitates the trigger expression of target genes and binds with a transcriptional activator. Movement of silkworm MBF1 from cytoplasm to nucleus during the developmental stage D3 binds with transcription factor BmFTZ-F1 (Liu *et al*., 2000). Li *et al*., (2009) quantified the MBF1 mRNA of silkworm *Bombyx mori* in tissue and stage-specific levels by using SYBR Green real-time RT PCR, results. The expression of MBF1 mRNA in all selected tissues such as the fat body, midgut, epidermis, and silk gland showed MBF1expression.The highest expression of MBF1 mRNA was observed in the silk gland; it indicates the relation of it with silk protein synthesis.

In addition to silkworms, many other organisms also demonstrated transcriptional co-activator activity by building a bridge between distinct transcription factors with sequence-specific DNA binding domains to TBP **(**De Koning *et al*., 2009), but did not bind directly to DNA. It appears to work by attracting TBP to promoters where transcription factors are located (Ozaki *et al*., 1999). In various organisms, the MBF1 has played an important role in stress response. Organisms like drosophila, yeast, and mammals showed induced expression of MBF1 under various oxidative stresses (Zou *et al*., 2000, Takemaru *et al*., 1997, Brendel *et al*., 2002). In Drosophila, exposure to paraquat strongly induces the expression of MBF1; leading to the generation of oxidative stress (Zou *et al*., 2000) and MBF1also plays a role in redox-dependent regulation of AP-1(activating protein-1) activity during oxidative stress (Jindra *et al*., 2004).

1.10. Pro-apoptotic genes

1.10.1. BmApaf-1-*Bombyx mori* Apoptotic peptidase activating factor1

BmApaf-1 is similar to mammalianApaf-1(Salvesen &Abrams, 2004; Wang and Gu, 2001) and showed high similarity with mammalian initiator caspases (Yang, 2015). Apaf- 1, protein homologues to CED 4 of *Caenorhabditis elegans*, was first identified from HeLa cells (Zou *et al*., 1997). Apaf-1 is a 130kDa protein, that consists of three distinctive domains (Zou *et al*., 1997) a caspase recruitment N-terminus CARD domain, a nucleotide binding and oligomerization domain (NOD), and a C-terminus two WD40 repeat domains. The CARD segment is the procaspase -9 binding domain, the NOD domain mediates apoptosome formation and the WD40 domain is the binding site for cytochrome c (Yuan and Akey, 2013). Eighty-five amino acids of the NH2 terminal exhibit homology to the CARD segment found within the prodomain of some caspases like CED-3, caspase-1, caspase-2, and caspase-9 (Thornberry *et al*., 1992; Yuan *et al*., 1993; Wang *et al*., 1994; Duan *et al*., 1996; Srinivasula *et al*., 1996a). Apaf-1 binds with caspase 9 only in the presence of cytochrome C and dATP in the cytosol.

Azaserine-induced oxidative stress and apoptotic and antiapoptotic gene expression were investigated by Muthangi, (2020), they revealed that both lower and higher doses of azaserine influenced the discrete alterations in the expression of the BmApaf-1 gene in different larval tissues of silkworm *Bombyx mori.* BmApaf-1 was upregulated against BmNPV infection and activates the mitochondrial apoptotic pathway (Wang *et al*., 2020). H_2O_2 induced oxidative stress in BmN-SWU1 cells of silkworm *Bombyx mori* ovary turn on a mitochondrial apoptotic pathway, related to this the BmApaf-1 transcript levels were increased (Chen *et al*., 2015).

1.10.2. BmICE-2–*Bombyx mori* Interleukin1β converting enzyme-2

The pro-apoptotic caspase gene *BmICE-2* consists of p20 and p10 domains with 284 amino acid residues, a catalytic site surrounded by a special sequence QACRG. It has caspase 9 proteolytic activities in E. coli as well as caspase 3 and caspase 9 activity in BmN-SWU1 cells (Yi *et al*., 2014). ICE-2 gene of silkworm *Bombyx mori* was subdued to sequence analysis and revealed that it consists of seven exons (Sun *et al*., 2010).

Rui-ting and colleagues checked the expression profile of *BmICE*, which significantly increased in the middle silk gland and posterior silk gland during the larval pupal transition (Chen *et al*., 2017). BmN-SWU1 cells of the silkworm *Bombyx mori* ovary react against oxidative stress induced by H_2O_2 by the upregulated expression of the BmICE gene which indicates the activation of the mitochondrial apoptotic pathway (Chen *et al*., 2015). The *BmICE-2* increases the rate of apoptosis in BmN cells (Zhang *et al*., 2013). At the end of the fifth instar larval stage, in the midgut during metamorphosis, the BmICE2 expression was highly increased (Franzetti *et al*., 2012). Ovary-derived cells of silkworm *Bombyx mori* were subjected to H_2O_2 and UV treatment and transcript levels of ice 2 and ice 5 genes were recorded. Ice 2 was more highly expressed than ice 5 after 0h and 5h H_2O_2 treatment. But after UV radiation ice2 was highly expressed at 0h, 1h, 2h, 3h, 4h, and 6h compared to H_2O_2 treatment (Sun *et al*., 2010).

1.11. Inflammatory response genes

1.11.1. TNFSF5-Tumor Necrosis Factor Superfamily 5

"TNF family ligands and their receptors play decisive roles in copious vital physiological processes including host defense, inflammation, apoptosis, autoimmunity, and immune system organogenesis". TNF super family members are death receptors and they are transmembrane proteins with three domains: an extracellular ligand binding domain**,** a transmembrane domain, and a terminal domain. Death receptors are involved in transmitting the death signal from the cell surface to the intracellular pathway upon substrate binding (Okouchi *et al*., 2007). In silkworm *Bombyx mori* two TNF superfamily members have been identified from chromosome 5 they are Bm3585 and Bm3614 (Zhang *et al*., 2010).

1.11.2. IL-6–Interleukin 6

"The pro-inflammatory cytokine IL6 produced by various cells including T and B Lymphocytes, Fibroblasts, Monocytes, Keratinocytes, Mesanglial cells, Endothelial cells, as well as different tumor cells"(Ataie-Kachoie *et al*., 2013). It has an active role in a variety of physiological processes, such as acute phase response, inflammation, mechanisms of host defense, hematopoiesis, and cellular growth (Kishimoto, 2006). IL-6 is also known as interferon β2 (IFN-β2), B cell stimulatory factor-2, and hybridoma/plasmacytoma growth factor (Ataie-Kachoie *et al*., 2014). Different inflammatory mediators including cytokines, prostaglandins, reactive oxygen species, and proteases are produced (Woodfin *et al*., 2010) by inflammation which initiates and accelerates the neutrophil trafficking to the inflammation site by IL-6 (Fielding *et al*., 2008).

Silkworm *Bombyx mori* ovarian cells exposed to TiO_2 nanoparticles induce oxidative stress and DNA damage which results in the upregulated expression of inflammatory response genes such as TNFSF5 and IL6. TiO_2 nanoparticles exposure conduces to the aggregation of peroxides and inflammation (Fang *et al*., 2022).

Thermal stress exhibits a significant loss to the sericulture industry by affecting the growth, development, survival, and reproductive success of silkworms. Thermal stress leads to the production of ROS, eventually, it exerts physiological and biochemical alternations in silkworms. Apoptosis can be induced by thermal stress, among other cellular stressors. The present study aims to investigate the expression of pro-apoptotic genes, transcriptional co-activators, and inflammatory response genes in silkworms subjected to thermal stress. By employing a multidisciplinary approach, including gene expression analysis by RTPCR, histological examinations, and biochemical assays are conducted to find out the influence of thermal stress on the physiological and biochemical activities of silkworms. The present study also proposed to identify the occurrence of apoptotic pathways in the silkworm *Bombyx mori* under thermal stress.

1.12. Objectives of the present study

❖ To analyze the genomic expression of transcriptional co-activators in the development of *Bombyx mori* under thermal stress.

❖ Gene expression analysis of pro-apoptotic markers in the development of *Bombyx mori* under induction of heat shock.

❖ Expression analysis of inflammatory response genes of *Bombyx mori* under thermal stress.

1.13. Relevance of the study

The Lepidopteran insect silkworm *Bombyx mori is* primarily used for the production of economically important silk, renowned as the backbone of the silk industry. In India, sericulture is an agro-based domesticated industry that plays an important role in the national economy, employment, and financial upliftment to the people who live in rural areas, especially as a primary source of income for women. The continuous domestication of silkworms leads to the loss of resistance to environmental alterations and they have become less resilient to environmental stress, particularly for temperature. As a result of global warming the high temperature significantly affects growth (Benchamin and Jolly, 1986), development, pupation, survival, reproductive success, biochemical and physiological alterations, etc. Ultimately thermal stress affects the quality and quantity of cocoons, leading to the loss of the sericulture industry. Corresponding to other cellular structures, heat stress can also persuade apoptosis (Chowdhury *et al*., 2006). In general, an ideal level of intracellular ROS is needed for the mechanism of cellular homeostasis. The body's inability to detoxify ROS results in the excess accretion of ROS, which finally brings about the formation of oxidative stress. Thermal stress leads to the production of ROS and causing oxidative stress. The complex mechanisms of thermal stress-induced apoptosis are not well understood in silkworms. A recent report demonstrated that H_2O_2-induced oxidative stress results in the activation of the mitochondrial apoptotic pathway in the silkworm *Bombyx mori* (Chen *et al*., 2015). The present study proposed to find out the influence of thermal stress on the expression of apoptotic-related genes, transcriptional co- activators, and inflammatory response genes.

Along with silk production silkworms have been used as a model organism in biological and scientific research. Silkworms have had tremendous applications in the various fields of sciences, research, and in engineering domains. However silkworms as well as silk materials used in wound healing, therapeutic drug discovery, tissue engineering, production of surgical meshes and fabrics, clinical trials, electricity and optical devices. In addition to this, silk gland proteins such as sericin and fibroin are used in cosmetics, pharmaceuticals, and medicinal applications (Chand *et al*., 2023). Recently biological researchers have given special attention to the biological properties of sericin and fibroin, such as "anticancer, antioxidant, cardio protective, gastro protective, anti diabetic, anti hyperlipemia and skin-health-ameliorating effects (Biganeh *et al*., 2022).

Apoptosis has an important role in the growth, development, and metamorphosis of silkworms (Li *et al*., 2010). So this study helps to find out the knowledge of apoptosis in silkworms in response to thermal stress, more over it also contributes to a better perception of apoptosis in other invertebrates and even vertebrates. Apoptotic study of silkworms helps to understand the potential disease-controlling targets affecting silkworms such as bacterial and viral infections. This work also helps to improve the silk quality by the expression analysis of silk synthesis-related transcriptional co-activators under thermal stress conditions. Expression analysis of the genes that involved in apoptosis, researchers can explore strategies to aid in breeding programs for developing silkworms with thermal resistance, disease resistance, and enhanced silk production traits. Some of the apoptotic-related genes of silkworm *Bombyx mori* show similarity with the mammalian apoptotic genes, which help to better, understand mammalian apoptosis and inflammatory response as silkworms used as a model organism. Silkworm apoptotic study in response to thermal stress can provide an improved understanding of the molecular mechanisms related to human diseases allied with irregular apoptosis, such as neurodegenerative diseases and autoimmune diseases.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Sericulture or silk farming is the process of breeding silkworms for the successful production of good quality and quantity of cocoons which chiefly focuses on improving the Indian economy. The formation of higher-quality cocoons is fundamental to accomplishing better silk production. Rather than better silk production, silkworms have been extensively used for several scientific research programs, biological applications, engineering domains, and medicinal fields, particularly for treatments, etc. To achieve all these purposes and for better quality silk production, the silkworms should be successfully reared by overcoming adverse environmental conditions, especially temperature. High-temperature exposure is stressful and potentially lethal for all insects. Silkworms are highly susceptible to temperature, which leads to the production of oxidative stress, and induces various physiological responses. This leads to alterations in growth, development, and reproduction, which are responsible for the incidence of diverse diseases and loss of cocoon production.

2.1. Silkworm as an Experimental Model

Silkworm *Bombyx mori* is categorized into different types of breeds based on the number of generations produced per year, they are univoltine, bivoltine, and multivoltine, hence it has been taken as a vital criterion for the scientific classification. Univoltine usually produce one generation per year, but bivoltine turn out two generations annually. In contrast multivoltine engender more than two generations per year. After egg laying univoltine eggs spontaneously enter the diapause stage, which is a period of arrested development characterized by reduced metabolic activity resulting in decreased oxygen consumption.

Bivoltine eggs show a facultative diapause mechanism while multivoltine eggs never enter the diapauses stage (Murakami and Ohtsuki 1989, Yamashita and Yaginuma 1991). Univoltine silkworms have the most extended larval life. They are less tolerant to environmental fluctuations especially slight variations in temperature and humidity but they produce high-quality cocoons once a year. However, compared to univoltine strains, bivoltine larvae produce better quality products and are much more endurable to adverse environmental conditions. While univoltine silkworms have short larval periods, they are more tolerant to environmental fluctuations but they produce tiny-sized cocoons. The heritable trait voltinism is normally governed by a set of genes and hormones, and the larval period is impacted by a series of environmental circumstances (Rao, 1998).

Silkworms have been utilized as a key factor in environmental monitoring and as an ecological indicator (Hamamoto *et al*., 2009). Because the growth and development of silkworms influence the change in environmental conditions (Rahmathulla, 2012) they are extremely sensitive to environmental pollution (Manasee Hazarika, 2017).

Sericulture predominantly focused on the production of good quality cocoons through the production of stress-resistant and disease-resistant good quality breeds. Rather than silk production, the scientific world focused on the other important qualities, characteristics, and uses of silkworms and applied them to the field of biological and scientific research. Because of the large size, effortlessness of rearing and management, other unique characteristics, and easiness of handling, silkworms have been utilized as a model organism in different research fields (Xia *et al*., 2014).

According to Chen *et al*., (2016), "silkworm has been used as a new laboratory animal model for human disease and drug screening". Silkworm *Bombyx mori* is considered a representative of the Lepidopteran order of class insecta, it has several features like "diminutive generation time, highly prolific nature, ease of handling, well defined genetic background and plentiful mutation resources", because of these characteristics silkworms used as a model insect, the qualities and characteristics of silkworms are used for new drug discovery for human diseases like "phenylketonuria, Parkinson's disease, Hermansky-Pudlak syndrome" etc. Meng *et al*., (2017) reported that silkworms have a tiny life cycle, well-understood genes with specifically sequenced genomes, and a large number of genes that are identical to silkworms, which makes them useful for different research areas.

According to the "structural, physical, chemical and mechanical properties of silk, it can be used in the field of research and engineering to make surgical meshes, fabrics, industrial and engineering materials, optical devices and electricity, drugs for wood healing, and it also used in pharmaceuticals and cosmetics"(Chand *et al*., 2023).

2.2. Impact of thermal stress on sericulture

Environmental conditions such as abiotic and biotic factors, among which temperature plays a major role in the successful growth of the sericulture industry through the successful production and growth of silkworms (Ueda *et al*., 1975, Benchamin and Jolly, 1986). Especially the fourth and fifth instar larval stages are more susceptible to elevated environmental temperature (Shirota, 1992; Tajima and Ohnuma, 1995).

Rahmatulla *et al*., (2004) observed that the growth and development of silkworms has a parallel connection with temperature and any ample changes in the temperature lead to detrimental effects on the growth and development of silkworm larvae. The modes of intensifying temperature harm physiological development as well as decreasing temperature also cause alterations in the physiology of silkworms.

The effect of temperature on the biochemical composition of hemolymph of larva and pupa was studied by Malik and Malik (2009), revealing that protein concentration was decreased with increasing temperature. An increase in free amino acid levels in hemolymph assists the protective role against thermal stress by increasing osmolarity and diminution in water loss by evaporation. Larva and pupa were homeostatically controlling their sugar levels in haemolymph. To prevent the irregular increase in osmotic pressure in the haemolymph during exposure to higher temperatures, the monovalent cations like Na+ and K+ were hyper-regulated, and divalent cations like Ca2+ and Mg 2+ were hypo-regulated.

Hussain *et al*., (2011) studied the effect of varying temperature and humidity on cocoon production. They conducted the study on eleven silkworm lines such as M-101, M- 103, M- 104, M- 107, Pak- 1, Pak- 2, Pak- 3, Pak- 4, PFI- 1, PFI-2, and S-1.They exposed the fourth and fifth instar larvae with different temperatures like 25±1, 30±1, and 35±1ºC. At high temperatures all the silkworm lines showed decreased cocoon parameters like cocoon weight, shell weight, cocoon shell ratio, filament length, filament weight and cocoon yield when exposed to 3 hr heat shock while the cocoon traits were increased at control temperature.

Hussain *et al*., (2011) investigated the effect of temperature and humidity deviation in the pupation, larval mortality and hatchability in fourth and fifth instar larva of eleven inbred silkworm lines such as M-101, M-103, M-104, M-107, Pak-1, Pak-2, Pak-3, Pak-4, PFI-I, PFI-II, and S-I. The fourth and fifth instar larvae were exposed to a temperature of 25, 30, and 35±1ºC. The impact of high temperature highly reflects the rate of pupation, hatchability and larval mortality. The control group (25±1ºC temperature and 70-80% relative humidity) showed an increased pupation, hatchability and decreased larval mortality while varying temperature and humidity decreased the pupation rate and hatchability and increased the mortality rate in all silkworm breeds.

Rahmathulla, (2012) reported that temperature directly affects all the physiological processes and development of silkworms. In his opinion, early silkworm larvae are invulnerable to environmental temperature and they can boost the survival rate of larvae and increase the quality of cocoons. The development of silkworms is directly correlated with temperature and any vacillation in temperature is catastrophic to the growth and development of silkworms. When temperature increases, the rate of all the physiological functions increases but a plunge in temperature leads to retarded physiological activities.

According to Kumar & Singh, (2012) alterations in the rearing conditions, especially temperature and humidity through larval rearing extensively influence the fecundity and fertility of silkworm moths. Upon exposure to high temperatures and low humidity, the silkworm moths lay fewer eggs. These changes lead to the unhealthy condition of larvae which will affect the pupation and development and ultimately it adversely affect the cocoon production. Due to external environmental stress, the silkworms require much energy to retain internal equilibrium. This study illustrates the changes in the rearing temperature and humidity negatively impacts the egg production and fertility of silk moths.

Li *et al*., (2014) compared the mid-gut gene expression profile of two silkworm varieties such as thermo tolerant variety ‘932’ and thermo sensitive ‘HY’ variety on exposure to high temperature. They spotted genes and metabolic pathways associated with heat stress and they revealed that high-temperature treatment exerts influence on some of the biological pathways embraced with oxidative phosphorylation, interconnected with glucose and lipid metabolism. Because of this reason, silkworms face difficulty ingesting the required quantity of fresh mulberry leaves. This study provides experimental proof for the differences between thermo tolerant and thermo sensitive silkworm varieties.

Wongsorn *et al*., (2015) studied the effects of temperature on growth, yield, and production of heat shock proteins in Eri silkworm. The larvae were treated with five different temperatures (36±1, 40±1, 42±1, 45±1 & 48±1ºC) and they observed that survival percentage, cocooning percentage, and most yield parameters reduced with higher temperatures, particularly in 42±1 to 48±1ºC. All measured parameters such as survival rate of larva, survival rate of larva to adult, and percentage of cocoon formation were recorded in the lowest value, exposure at 48±1ºC. This study suggested that high heat shock creates a negative impact on the growth, survival, and cocoon production.

Tanjung *et al*., (2017) examined the effect of heat shock treatments on the growth and development of silkworm larvae. They experimented with exposure to four different temperatures such as 34, 38, 42ºC and an ambient temperature, and revealed that heat shock can increase the percentage of mortality, expedite the larval stage, and also diminish larval weight by reducing the growth of larva, food consumption, and digestibility leads to the non-fulfillment of cocoon formation, pupae, and imago. Ultimately the effect of heat shock declined productivity.

Sun *et al*., (2017) scrutinize the ‘effect of transient high-temperature treatment (THTT) on the intestinal flora, and the relation between intestinal flora and the gene expression in silkworms was investigated by changes of the intestinal flora at 48, 96, and 144 h following transient high-temperature treatment of 37ºC for 8h’. Followed by principal component analysis, Enterococcus, and Staphylococcus abundances showed a negative connection with other prominent genera. After THTT, activation or suppression of the Toll and RNAi pathways depended on the increased as well as diminished expression of spatzle-1 and dicer-2 genes. They also investigated the effect of THTT on silkworm intestinal bacteria and the relationship between the genes related to innate immunity, cell cycle, and apoptosis by using qRT- PCR. The samples HT-48, HT-96, and HT-144 h were treated with high temperature, and quantified the expression of genes associated with innate immunity, cell cycle and apoptosis and revealed that ‘Bmovo, Bmdpp, Bmyki, Bmiap, Bmdelt, Bmtal, BmMyc, BmSTAT, BmPGRP-LE, BmPGRP-LB, BmSOCS2, and Bmdrc-2 were not significantly different among HT- 48 and HT-96 but these genes showed highest level of expression in HT-144'. This study provides a novel idea about the relationship among THTT, intestinal flora, and host gene expression.

Xiao *et al*., (2017) confer that transcriptome profiling of two different silkworm strains such as thermal sensitive and thermal resistant was subjected to high temperature and humidity up to 48 hours of post-exposure and they investigated the time gap between molting of strains along with larval deaths to evaluate the heat and humidity tolerance. They discovered a total of 4,944 differentially expressed genes (DEGs) of which 4,390 were interpreted and 554 were new. 'Gene ontology (GO) analysis of 747 DEGs between RT 48h (Resistant strain with high-temperature treatment for 48 hours) and ST 48 h (Sensitive strain with high-temperature treatment for 48 hours) exposed major enhancement of 12 GO terms, including metabolic process, extracellular region, and serine-type peptidase activity.' Twelve newly determined DEGs that may be involved in the heat-humidity stress response in silkworms. This study provides awareness about the role of genes and other biological processes related to high humidity and temperature tolerance.

Mir & Qamar, (2018) studied the effect of thermal stress in the silkworm *Bombyx mori* and they revealed that the vitality, body weight, and shell percentage of the cocoon were markedly decreased in response to high temperature and promoted the expression of stress-related genes and CSP (chemosensory protein) genes.

Tanjung & Lenny, (2019) reported the application of heat shock for 3 hours at 42ºC in IVth instar larvae of *Bombyx mori*, then the larvae were rearing up to cocoon formation. The pupae underwent physical and chemical examination revealed that heat shock affects the pupal formation and pupae become irregular in shape with black colour. Chemical analysis showed that the total fat and carbohydrate content was adversely affected, but the protein concentration does not show any significant reduction on exposure to heat shock.

Li *et al*., (2019) emphasized that high-temperature treatment shortened the fifth instar larval duration, and the time to reach maturity was increased. The larvae were treated at two different temperatures such as 28ºC and 36ºC; at high temperatures, the 20E hydroxy ecdysone is upregulated in haemolymph and its transcription levels of 20 E response genes and synthesis-related genes were upregulated. Their result revealed information about the phosphorylation of Akt and regulation of metamorphosis of lepidopteran insects by the activity of the CncC/Keap 1 pathway.

Chakraborty and Dastidar (2022) studied the effect of temperature on egg, larvae, pupae, and adults of multivoltine White Nistari race. They were subjected to thermal treatment at 18 to 44ºC during three consecutive days with 1hr 30 min. The fourth instar larval haemolymph produced a 72 kDa protein band after increasing the temperature to 44ºC, besides that cocoon and shell weight also increased significantly.

2.3. Antioxidant enzyme activity

Nabizadedh and Kumar, (2010) studied the effect of two different high temperatures 35±1ºC and 40±1ºC on the level of activity of CAT on larvae of five bivoltine breeds such as CSR_4, JROP, KA, CSR_2, and NB_4D_2. They investigated the activity of catalase on three different body tissues such as fat body, midgut, and haemolymph, and they revealed that, compared to midgut and haemolymph the fat body showed the highest level of activity when they were exposed to thermal stress. Although compared to five bivoltine breeds CSR_2 showed a greater level of catalase activity under the control and thermal stress exposed groups. This study proved that the effect of high temperature influences the activity of CAT enzymes. Nabizadedh and Kumar, (2011) also pointed out that catalase activity in the fat body showed a positive correlation with control and high-temperature exposed groups. Compared to CSR_4, JROP, KA, CSR_2 and NB_4D_2, the CSR_2 breed showed a high level of catalase activity and an ability to withstand thermal stress. This work gives an idea about 'select thermo tolerant breeds and helps to develop resistant hybrids for tropical areas'.

Jena *et al*., (2013) experimented on pro-oxidants and antioxidants under thermal stress conditions of male pupal testes of tropical tasar silkworm *Antheraea mylitta.* Furthermore, they studied the effect of temperature on the physiological activity and rate of oxygen consumption. The male pupae treated with high temperatures (40±1ºC) showed elevated levels of thiobarbituric acid reactive substances and total hydroperoxides. Correspondingly the study showed that, following high temperature the male pupal testes have greater levels of antioxidants such as SOD, CAT, GST, ascorbic acid, and low molecular thiols on moderate exposure to temperature. The mechanism of activation of these physiological reactions assists in alleviating the reactive oxygen species formed during thermal stress. Compared to moderate temperatures the oxygen consumption rate was increased in the higher temperature and there is a correlation between oxygen intake, oxidative stress, and antioxidant activity. These findings suggest that *Antheraea mylitta* regulates antioxidants in response to thermal stress.

Li *et al*., (2018) reported that treatment of TiO_2 NPs (Titanium dioxide nanoparticles) has the capacity to alleviate the oxidative stress formed by the high temperature of 30ºC. This is a new insight against the oxidative stress originated by thermal stress at 30ºC. Economically relevant insect silkworms are receptive to unfavorable environmental changes and extremely sensitive to temperature variation. The effect of temperature decreases the accurate cocoon production. To alleviate the oxidative stress by inducing TiO_2 nanoparticles can reduce cocoon damage; it also helps to improve silkworm body weight by around 6%, ROS levels in the fat body were decreased and expression levels of heat shock protein HSP 70, antioxidant enzymes SOD, CAT and TPx were increased their fold expression.

Kumar and Michael, (2019) studied the effect of different stresses like cold, hypoxia, nuclear poly necrosis virus on the GPx activity of fourth and fifth instar larvae of silkworm *Bombyx mori*. Upon exposure to different stressors, the GPx activity of fourth and fifth instars larval tissues was increased while upon recovery from stresses the antioxidant activity was returned to the normal value.

2.4. Heat shock protein- HSP70

Velu *et al*., (2008) used the semi-quantitative reverse transcriptase polymerase chain reaction (RT PCR) approach to investigate the expression of heat shock protein genes concerning temperature stress. The silkworm strains Pure Mysore, Nistari, and NB4D2 were subjected to thermal treatment at 38±1ºC and 42±1ºC / 1 hr, and allowed 2hr recovery time. They test out the expression of Hsp genes such as Hsp 70, 40, 20.4, and 20.8, the results illustrate that Hsp genes showed a significant variation in the expression pattern. Compared to small heat shock proteins, large heat shock proteins such as HSP70 and HSP40 showed increased expression, through the recuperation time compared with 1 hr thermal treatments. The Hsp 70 demonstrated higher expression while Hsp 40, 20.4, and 20.8 genes showed increased expression at initial stages and then decreased during the recuperation time. However, in the tissue-specific expression, Hsp 70 showed higher expression in the midgut and fat body than the cuticle and silk gland. This experiment reveals the importance of heat shock proteins against thermal stress.

Li et al., (2012) conducted an experiment to compare the expression of inducible heat shock proteins in heat-treated silkworms. The study focused on genetic as well as non genetic factors influencing the expression of heat shock proteins in silkworms. The study points out that small heat shock proteins HSP 19.9 and 20.4 showed increased expressions and decreased expression of HSP70 in different tissues of heat-treated larvae. The study also revealed higher variations in the expression of small heat shock proteins and HSP 70 under different conditions such as heat exposures and genetic backgrounds.

Li *et al*., (2014) reported that, when the thermo tolerant variety '932' and thermo sensitive variety 'HY' were exposed to high temperatures shows a deviation in the expression of heat shock genes. During the early period of incessant heat stress, different HSP genes are upregulated initially but down regulated after 24 hours. The mRNA quantity of several HSPs shows higher in thermo tolerant '932' strain, and HSP 70 shows down regulated expression compared to the sensitive variety. HSPs and their level of expression may indicate the capacity to fight against extreme temperatures. This work provides information regarding the thermo-tolerant differences between different strains of silkworms.

Silkworm *Bombyx mori* cell lines (BmN4, BmN-FK, and BmN4SID-1) were treated at high (45ºC for 35 minutes and 24 h cultures), and low temperatures (0ºC each for 3&7d cultures), and the work focused to examine the expression of genes related with cell death and temperature using semi-quantitative PCR. At low temperatures, dsHsp70 treatment repressed the gene expression and decreased the cell viability of treated cells (Kim *et al.*, 2015).

Yu *et al*., (2015) stated that the main role of HSP70 is a molecular chaperone, rather than this feature it takes part in two major functions, 'cellular development and caring of living organisms from environmental traumas such as heat, drought, salinity, acidity, and cold'.

Hsp70s (heat shock protein 70s) are a class of molecular chaperones that are highly conserved and ubiquitous in organisms ranging from microorganisms to plants and humans. Most research on Hsp70s has focused on the mechanisms of their functions as molecular chaperones, but recently, studies on stress responses have come to the forefront. Hsp70s play key roles in cellular development and protecting living organisms from environmental stresses such as heat, drought, salinity, acidity, and cold. Moreover, functions of human Hsp70s are related to diseases including neurological disorders, cancer, and virus infection. In this review, we provide an overview of the specific roles of Hsp70s in response to stress, particularly abiotic stress, in all living organisms.

Mousavi *et al.*, (2017), experimented to find out the expression pattern of HSP70 and HSP90 genes in various silkworm hybrids (Two Japanese maternal parents and two Chinese maternal parents) exposed to severe heat shock at 45ºC for 35 minutes. The fat body was selected as the sample and it was collected after 0, 2, 4, and 24h after thermal treatment. Compared with the control (24ºC), the extended treatment at 8h at 39ºC showed upregulated expression of both HSPs. After heat exposure both HSPs significantly expressed, Japanese female parents were more sensitive to heat shock, concerning increasing time, expression was decreased.

Guo *et al.*, (2018) conducted an experiment to find out the effect of variation in the ambient temperature in Dazo silkworms at 13ºC, 25ºC, and 37ºC. They revealed that heat shock proteins play an important role in both low and high temperatures. In this study, the heat shock proteins hsp70A1, hsp70A2, and hsp70A3 showed increased expression in silkworms after low and high-temperature exposure.

Punyavathi and Manjunatha (2020), investigated gender sensitivity to ambient heat in young and late larval instars of silkworm *Bombyx mori*. Following different thermal stress, various instars of male and female larvae revealed differences in the expression of heat shock proteins HSP70 and HSP 90. Male larvae showed prudent expression of HSP70 while female larvae had elevated levels of BmHsp70 and BmHsp90 transcripts in response to heat shock treatment. Exposure at 40ºC, the female larvae successfully spun cocoons with increased weight and silk content while exposure at 45ºC observed autophagy leading to larval death. This study draws attention to sex-specific and preferential actions of HSPs on the survivability and biosynthetic potential of *Bombyx mori* larvae during different instars.

According to Fang *et al*., (2021), a comprehensive investigation of the silkworm *Bombyx mori* whole genome revealed the response of the heat shock protein 70 families to various environmental stresses including heat, cold, and pesticides. Regarding this study, HSP70-1, HSP70-2, and HSP70-3 were significantly up-regulated in response to heat exposure at 37ºC and 42ºC as well as cold stress at 2ºC.

2.5. Transcriptional co-activators in silkworm

2.5.1. *Bombyx mori* Yorkie (BmYki gene)

The genes associated with the Hippo signaling pathway in silkworm *Bombyx mori* were recognized by Qian *et al*., (2013). In silico cloning or RT-PCR were used to attain the sequences of the genes associated with the Hippo signaling pathway, they are BmHop, BmSav, BmMats, BmWts, and BmYki. Compared with the other species' genome sequences, assumed amino acid sequences of the genes were identical to other species' genomes which emphasize the function of the Hippo signaling pathway in silkworms alike to other species.

Silkworm *B. mori* was subjected to several experimental examinations by Zeng *et al*., (2017) to find out the expression and biological role of transcriptional co-activator BmYki gene. This study revealed an idea about the location of the BmYki transcripts; they are ubiquitous and spotted in most of the selected tissues and developmental stages but not plentiful in all located regions. On the third day of the Fifth instar, the larval silk gland recorded the highest expression. BmYki facilitates the transcription of genes related to cell proliferation and apoptosis; it functions as a regulator of organ growth-related biological processes. It has an important role in silk protein synthesis.

Poikilothermic insect silkworm is sensitive to environmental temperature, the impact of high temperature on the flora present in the intestine of silkworms and the connection between intestinal flora and gene expression concerning transient high-temperature treatment was done by Sun *et al*., (2017). After transient high-temperature treatment, HT 48(h) and HT 96(h) samples showed down regulated expression of the BmYki gene compared to respective controls in the midgut. This observation pointed out that during high-temperature treatment Hippo signaling pathway was repressed in the midgut which causes suppression of growth and development of the midgut, finally leading to the diminishing of the intestinal bacterial count.

Zeng *et al*., (2018) revealed that BmYki is transcribed into four functional splicing isoforms in the silk gland of silkworms and they act as regulators of Yki target genes and also give away some diverse sequencing on the expression of Yki target genes. This study yields an idea regarding the role of BmYki isoforms in silk protein synthesis.

Liang *et al*., (2018) studied the obligations of the Yki gene in the silkworm *Bombyx mori*; they focused on the BmYki3 gene, a core component of the Hippo signaling pathway. They reported that BmYki3 expedites the process of cell migration and cell division. It also extends cultured cell and wing disc size. They also recommended that the growth of the silk gland and mid gut be controlled by the Hippo signaling pathway by the elevated expression of the BmYki3 component.

In silkworms the transcriptional co-activator BmYki has seven alternative isoforms; they have diverse transcriptional co-activator activity reported by Liang *et al.,* (2019). In this study, they infer that BmYki 3 assists the process of cell migration and cell division and also helps to expand the size of cultured cells and wing discs. They also detected certain peculiar characteristics of alternative isoforms of BmYki such as cell size, location in the cell, cell migration, cell cycle, and transcriptional co-activator activity. The gene expression study revealed that cell cycle and apoptosis are controlled by over-expressing BmYki.

Yin *et al.,* (2020) investigated the role of the BmSd gene in u11 and p50 strains of silkworm *Bombyx mori*, BmSd is one of the components of the Hippo signaling pathway. BmYki is the transcriptional co-factor of BmSd and BmSd is the first transcriptional partner of BmYki. The hippo signaling pathway controls wing disc size in silkworms. BmYki and BmSd expression was down regulated in the u11strain compared to the p50 strain. Decreased expression of BmSd leads to the down regulation of BmYki and BmSd interaction, resulting in developmental defects in the wing of the u11 strain.

2.5.2. Multiprotein Bridging Factor 1(MBF1 gene)

Li *et al*., (1994) isolated the MBF1 and MBF2 and he proposed the bridge model. MBF1and MBF2 form a hetero dimer structure with 18 and 22-kilo daltons (kDa) polypeptides and serve as a bridge between BmFTZF1 and TATA Box binding (TBP) protein and mediate transactivation by stabilizing the protein DNA interaction in silkworm *Bombyx mori*. BmFTZF1 is a homologue of Drosophila FTZF1 (Fushi-tarasu). MBF1 and MBF2 can't bind directly to the DNA. BmFTZF1 DNA complex becomes stable after the binding of MBF1 to BmFTZF1.

Another model for activating transcription through MBF2 direct contact with TFIIA was proposed by Li *et al.,* (1997). MBF1 forms a bridge between FTZF1 and TBP, MBF1 interact with MBF2 and MBF2 directly connected with TFIIA and this interaction consents to transcriptional activation.

Transcriptional coactivator MBF1 quantification in silkworms was done by Li *et al*., (2009) using real-time RT-PCR. They isolated the total RNA from the fifth instar larval silk gland, midgut, fat body, and epidermis. They recommended that MBF1 mRNA be presented in all selected tissue samples and great expression was reported in the silk gland indicating the role and importance of the MBF1 gene in the production of silk proteins.

2.6. Pro-apoptotic genes

2.6.1. *Bombyx mori* Apoptotic peptidase activating factor1(BmApaf-1gene)

Chen *et al.,* (2015) studied the apoptosis process in silkworm ovarian cell lines by the effect of oxidative stress induced by hydrogen peroxide. They analyzed the ROS level, mitochondrial response after oxidative stress, release of cytochrome c, and expression of genes related to apoptosis. Apoptosis-related gene BmApaf-1 upregulated in H_2O_2-treated BmN-SWU1 cells which are analyzed by using quantitative real-time PCR.

Azaserine-induced oxidative stress leads to the upregulated expression of the BmApaf-1 gene in the midgut and fat body and a down regulated expression in the posterior silk gland. Although the 5mM azaserine induction increased the expression of BmApaf1 in the fat body (Muthangi, 2020).

Ye *et al.*, (2023) examined the effect of a sub-lethal dose of chlorantraniliprole in silkworms, this study revealed that exposure persuades autophagy and apoptosis in midgut by distracting the calcium equilibrium. During apoptosis, Apaf-1 expression is upregulated in the midgut through the increase of intracellular calcium release which leads to mitochondrial damage.

2.6.2. *Bombyx mori* Interleukin1β converting enzyme-2 (BmICE-2gene)

An examination was conducted on the basement of apoptosis in the silkworm *Bombyx mori* was done by Zhang *et al*., (2010). They identified fifty-two apoptosis- related genes; BmICE-2 was one member of the caspase family of genes with eight exons. Sun *et al*., (2010) explore the function of two caspase family genes ICE-2 and ICE-5 in the apoptosis of *Bombyx mori* cells during the induction of hydrogen peroxide and UV irradiation. They analyzed the morphological features of treated cells and deviation in the mitochondrial membrane potential. Both genes participated in the apoptosis process with various expression patterns for both treatments.

Compared to UV-induced apoptosis H_2O_2 treated apoptotic cells showed a very sudden and prominent deviation in the mitochondrial membrane potential. Both ICE-2 and ICE-5 were involved in the apoptotic process, after H_2O_2 treatment ICE-5 expression was elevated than ICE-2 while UV-induced apoptotic cells showed higher expression of ICE-2 than ICE-5. Both gene expression patterns revealed that the mitochondrial apoptotic pathway is active in *Bombyx mori* under stress conditions.

Yi *et al*., (2014) spotted a potential new pro-apoptotic property-carrying gene with a polypeptide of 284 amino acid residues characterizing a sequence^{169} QACRG^{173} surrounded the catalytic site with a p10 and p20 domain. BmICE-2 plays an initiator caspase role in the apoptosis of BmN-SWU1 cells.

To investigate the function of BmICE-2, Yi and Ma (2017) constructed a BmICE-2 interference vector; the interference target sequence was added to micro RNA and studied the effect of interference. In BmN-SWU1 cells, the BmICE-2 expression was significantly inhibited at mRNA and protein levels by interference vector. This study also endowed with a mechanism behind the control of silkworm cell apoptosis by BmICE-2.

During metamorphosis, differentiated the expression levels of the BmDredd gene in the middle and posterior silk gland of silkworms with apoptosis- related effector caspase BmIce (Chen *et al*., 2017). Quickly upregulated expression of BmIce beginning at 24 hr post spinning and attained greatest expression at 48 hr post spinning. Silk gland apoptosis starts from larval to pupal transition state, in this stage posterior silk gland showed upregulated expression of BmIce. According to their findings, the expression level of BmIce is significantly upregulated in the middle and posterior silk glands.

2.7. Inflammatory response genes

2.7.1. Interleukin 6 (IL-6 gene) and Tumor Necrosis Factor Super Family 5(TNFSF5 gene)

A genomic level examination of apoptotic-related genes in silkworm *Bombyx mori* was conducted by Zhang *et al*., (2010) made use of nucleotide and protein Blast searches and they recognized tumor necrosis Superfamily 5 (TNFSFS5). Exposure of TiO_2 nanoparticles in the silkworm *Bombyx mori* ovarian cells (BmN) leads to the production of oxidative stress causing cell damage studied by Fang *et al*., (2022). More than 20 mg/L H_2O_2 induction results from the significant toxic effect on cell viability and DNA damage by the accumulation of ROS. Additional genomic level analysis revealed that important inflammatory response genes such as IL6 and TNFSF5 expression were increased significantly resulting in inflammation. This study revealed induction of TiO_2 nanoparticles causes the formation of inflammation in silkworm *Bombyx mori* cells.

Zhang *et al*., (2021) treated silkworms with hydrogen sulfide (H_2S) and investigated the variation in the gene expression of the fat body of silkworms by comparative transcriptome analysis. This study revealed that H_2S treatment is beneficial for silkworm growth and development. H_2S induction decreases the expression of inflammatory response genes and inhibits inflammation in silkworms. The TNF signaling pathway is down-regulated in the silkworm fat body after being treated with H_2S.

2.8. Caspase3 gene

During starvation, the brain neuronal apoptosis of silkworm larvae was studied by Kim *et al*., (2012), focused on the function of Foxo transcription factors and the role of PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways in apoptosis of brain neurons. Under starvation, the activation of caspase3 indicates the process of apoptosis in the neuronal cells of the brain by the inhibition of cell proliferation.

Hu *et al*., (2018) conducted a study to find out the apoptosis of the posterior silk gland occurred during the spinning period and the function of the PI3K/Akt signaling pathway in the process of apoptosis. In this study, the morphological characters of the silk gland spectacularly changed with a declined expression of PI3K/Akt pathway-related genes and increased expression of apoptotic-related gene caspase3 which eventually reduced the synthesis of fibroin. Upregulated expression of caspase3 gene during the spinning period which reflects the occurrence of apoptosis in the posterior silk gland. Rather than the transcription level of the caspase3 gene, the enzymatic activity also showed an up-regulated trend along with the spinning period.

Programmed cell death (apoptosis) is controlled by the independent direction of genes. The importance of the *Bm30kc6* gene in cell apoptosis and the signaling pathway of silk gland degradation were investigated by Xiao *et al*., (2020). Cell apoptosis transpired by UV treatment, and excess expression of the *Bm30kc6* gene leads to diminished expression of the caspase3 gene. Meanwhile, the suppression of Bm30kc6 by small interfering RNA results in the upregulated expression of caspase3 and other apoptosis-related genes.

Ye *et al*., (2023), explore the harmful effect of exposure of a sub lethal dose of chlorantraniliprole on silkworm *Bombyx mori.* In BmN cells chlorantraniliprole exposure (0.02mg/L) is a major factor for releasing intracellular calcium and although 0.01mg/L CAP exposure leads to damage to mitochondria, rupture of the nuclear membrane, condensation of chromatin, and increased in autophagy and apoptosis-related genes. Consequently CAP exposure is a reason for the elevated expression of caspase 3gene in silkworm. These findings accentuate the significance of considering the potential environmental impact of pesticide use and necessitate for strategies to curtail their damaging effects on non-target organisms.

The above review o literature focused on the impact of thermal stress on sericulture, heat shock protein gene, expression of apoptotic related genes, transcriptional co-activators, inflammatory response genes, and caspase3 gene in silkworm, *Bombyx mori.* The high temperature negatively influenced the growth and development of silkworm; physiological alterations have been affected due to thermal stress. Thermal stress also altered the expression of genes associated with apoptosis in silkworm *Bombyx mori*.

2.9. Histopathology of silkworm midgut

Silkworm larvae fed with *Bacillus thuringiensis israelensis* leads to the destruction of midgut epithelium, vacuolation in the cytoplasm, breakdown of microvilli, nucleus necrosis, and dislocation of cytoplasmic organelles were reported by Mathavan *et al*., (1989). Midgut cell damage and karyopyknosis was reported by Su *et al*., (2014). Wang *et al*., (2015) reported that the silkworm midgut cells were damaged under the exposure of phoxim insecticide. Basal lamina of midgut disappeared and occurrence of karyopyknosis was noticed in response to insecticide exposure. Impact of thermal stress on the structure of midgut was studied by Sun *et al*., (2022). They exposed the fifth instar silkworm larvae to high temperature of 34±1ºC for 72 h, and maintained a control group at 25±1ºC. The silkworm midgut was collected and stained with hematoxylin and eosin by following the routine procedure. In this study they revealed that the morphology of midgut was changed with irreparable damage in response to high temperature. High temperature exerted injury in the epithelial cells of midgut; intestinal cells were arranged more loosely with a disordered manner. Midgut cell vacuolation has occurred in the heat treated group.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Experimental organism

Scientific position

Kingdom : Animalia

Phylum : Arthropoda

Class : Insecta

Order : Lepidoptera

Family : Bombycidae

Genus : Bombyx

Species : mori

Scientific name : *Bombyx mori* L.

The present investigation was carried out in diverse life stages of silkworm *Bombyx mori* L. Affiliated with the Phylum Arthropoda, Class Insecta and Order Lepidoptera, Family Bombycidae. The life cycle of silkworm passes through the diverse developmental stages such as egg, larva, pupa and adult, the larval stage undergoes consecutively with five instar stages and four moulting stages. After completing about four week's time of larval stage, the larvae take on spinning and it forms a basic cocoon frame with around 72 hrs of time period. The initial morphological structures of pupa formed within another two or three days and the adult emerges after completion of pupal period about 12 days. Adult's start mating immediately and lay eggs within four days of maturation.

3.2 . Rearing and management of Silkworm

3.2.1. Collection of material

The experimental organism CSR 26 silkworm DFLs were procured from Central Sericultural Research and Training Institute (CSRTI) Mysore, and were reared under laboratory conditions. The Rearing room and all the equipment used for rearing were disinfected with bleaching powder and 2% formalin which lend a hand to keep away from pathogenic infection from the surrounding environment and equipment.

3.2.2. Management and Incubation of DFLs

The collected DFLs were incubated under 25ºC for up to ten days in our laboratory, the eighth or ninth day after oviposition the embryos developed black eye spots on the eggs, this pinhead stage, "the embryos were more sensitive to light". For hatching simultaneously, the DFLs were kept in black boxes up to the expected day of hatching. On the day of hatching DFLs were incubated in between 25.5ºC to 26.5ºC. Then the eggs were exposed to light and when hatching was noticed on the egg card, within two hours, hatching was completed (Krishnaswami, 1978; Datta, *et al.,* 1996).

3.2.3. Rearing of silkworm

The newly hatched larvae were transferred from egg card using a fine mesh and then the larvae were fed with freshly chopped tender mulberry leaves. The first instar and second instar stages(young age) were maintained under 27-28ºC temperature and 85-90% relative humidity, with 2-3 feeds per day, clean wet sand trays were placed near the rearing trays to make sure of the humidity. Within 3-4 days the first instar larvae were uniformly completed their first moult. Each moulting was completed within 24 hrs. Cleaning was done rapidly after resumption from first moult, with a fine mesh spread over the rearing tray before feeding.

Second moult ensue within the next 2 or 3 days. Third to fifth instar is known as the late age of silkworms. The late age rearing maintained with 25±2ºC and 75±5% relative humidity. Both third instar and fourth instar larval period completed within 3-4 days. Normally the fifth instar stage lasted for about 6-8 days, in this stage larvae were fed with fully matured mulberry leaves. After completing fifth instar larval stage, the fully grown silkworms were shifted to mountages for spinning cocoons (Krishnaswami, 1978; Datta, *et al.,* 1996). Spinning and cocoon formation completed within 3-4 days, then the larva transformed in to pupa within the period of 8-10 days. Then pupa transformed in to adult and lasted for 3-4 days.

3.3. Experimental design

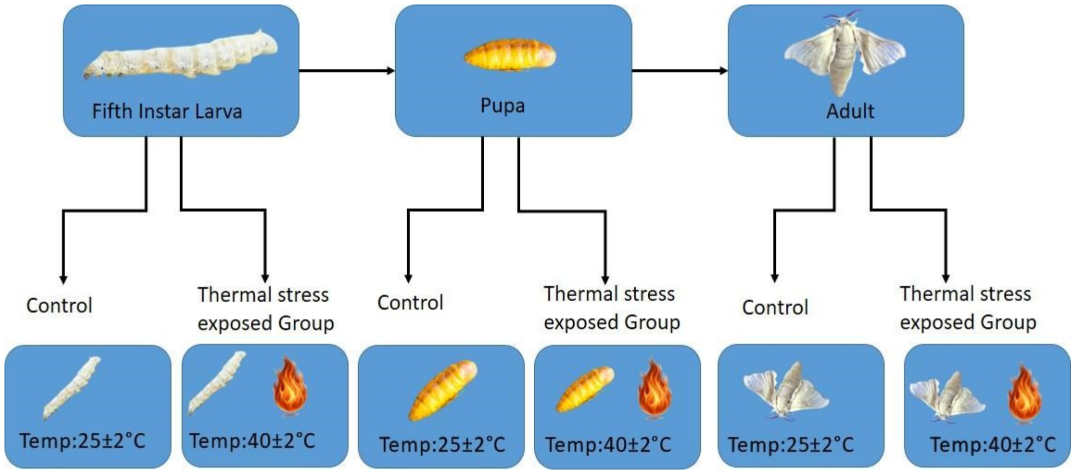
The present work was carried out in the fifth instar larva, pupa and adult stages. After the fourth moult the fifth instar larvae were grouped into two groups, control(C) group and thermal stress exposed (T) group. The group 1 was treated with normal rearing temperature about 25±2ºC and the group 2 larvae were treated with 40±2ºC temperature in 1 hr per day during the fifth instar stage using thermostat incubator (Rock plus Model). The treatments were also continued during the pupal and adult stage.

Experimental Design

Group 1: C – Control

Group2: T-Thermal stress exposed

Figure 3.1. Experimental Design



3.4. Analysis of morphometric parameters of developmental stages

3.4.1. Larval weight

Ten larvae were randomly selected from each group on the first day to sixth day of fifth instar larva, and were weighed by using an electronic balance (SCALE TEC-SAB203, Model- ATY224) and calculated the average weight gain of each day with six replications.

3.4.2. Pupa and Adult weight

Average weight of pupa and adult were examined in both control and thermal stress exposed group, in this approach randomly selected ten pupae and adults were used. Average weights of adults were examined by sex specific manner.

3.4.3. Larval length

Sequential gains of average length (cm) of first day to sixth day of fifth instar larvae were examined manually by using scale and graph paper. Ten larvae were randomly selected for the experiment from each group with six replicas.

3.5. Dissection and collection of tissues

Healthy fifth instar larvae, pupae and adult were randomly chosen from two groups and were dissected with 0.75% normal saline solution. Silk gland, midgut and fat body of fifth instar larvae, fat body of papa and adult were dissected out, washed in ice cold saline solution and weighed in electronic balance. Then a 10% homogenate of the tissue samples were prepared in 50mM phosphate buffer (pH7.4) solution used with an ice cold sterile mortar and pestle. The homogenized tissue samples were centrifuged (Remi- CM 12 PLUS, Thermo scientific Sorvall ST 8R) at10,000 RPM for 20 minutes at 4ºC, and the supernatants were collected in pre-chilled vessels and stored at -20ºC (Low temperature freezer Labline-VDF-2851-S) for diverse biochemical experiments (Sahoo *et al*., 2015).

3.6. Mortality rate

Throughout the experiment of fifth instar larval treatment, dead and paralyzed larvae diligently take away from each group. Subsequently, the number of dead larvae from each group was quantified in order to determine the mortality rate. The percentage mortality of larvae in their fifth instar was then calculated using the formula established by Megalla (1984).

$$\text{Mortality percentage} = \frac{(Number\ of\ dead\ larvae \times 100)}{Total\ number\ of\ larvae}$$

3.7. Biochemical Analysis

3.7.1. Estimation of Malondialdehyde (MDA)

MDA was estimated by the method of Ohkawa *et al*., (1979).

Reagents

1. 1.15% Potassium chloride (KCl)

2. 8.1% Sodium dodecyl sulphate (SDS)

3. 20 % Acetic acid - pH of the 20 % acetic acid solution was adjusted to 3.5 with sodium hydroxide (NaOH).

4. 0.8% Aqueous solution of thiobarbituric acid (TBA)

5. N-Butanolpyridine (15:1V/V)

6. 1, 1, 3, 3 Tetra methoxy propane (TMP) used as standard.

Procedure

1. 1 g tissue was homogenized in 9 ml of 1.15 % KCl, centrifuged at 1000 g for 10 minutes at 4ºC and supernatant was collected.

2. 0.2 ml 8.1 % SDS, 1.5 ml 20% acetic acid (pH 3.5-Labtronics -10) and 1.5 ml of aqueous solution of TBA were added to 0.2 ml of tissue homogenate.

3. The mixture was made up to 4ml with distilled water and then heated in a water bath at 95ºC for 60 minutes.

4. Then cooling under tap water, 1ml of distilled water and 5ml of N-butanol pyridine mixture were added and shaken vigorously.

5. After centrifugation at 4000 RPM for 10 minutes. Organic layer was taken and its absorbance at 532 nm was measured against a butanol blank.

6. TMP is used as internal control.

7. Lipid peroxidation level was expressed as Malondialdehyde produced in mM/g protein.

3.7.2. Estimation of total soluble Protein

Estimation of total soluble protein concentration was done by using Lowry's method (Lowry *et al.,* 1951)

Reagents required

A. 2% Sodium carbonate (Na_2CO_3) in 0.1N Sodium Hydroxide (NaOH)

B. 1% Sodium Potassium Tartrate (Na-K) in water (H_2O)

C. 0.5% Copper Sulphate ($CuSO_4.5H_2O$) in H_2O

D. Reagent I:48 ml of reagent A,1 ml of reagent B, 1ml of reagent C

E. Reagent II-1 part Folin-Phenol [2 N]: 1 part distilled water

F. BSA Standard- 10 mg BSA dissolved in 100ml of distilled water

Procedure:

Preparation of standard curve was done by using five known concentration of (0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1 ml) BSA working standard. Experimental samples were taken in test tubes and were made up to 1 ml using distilled water. Then Lowry concentrate (Reagent I) 4.5 ml was added and incubated for 10 minutes. After that 0.5 ml Folin's reagent (Reagent II) was added and kept for 30 minutes at dark. The test tube with 1 ml of 0.1N NaOH was considered as blank. The optical density of the sample measured at 650 nm using a UV spectrophotometer (Systronics-AU-2701).

$$Concentration\ of\ unknown = \frac{OD\ of\ unknown}{OD\ of\ standard} \times Concentration\ of\ standard$$

3.7.3. Catalase (CAT)

Catalase enzyme activity determined by using the method of Luck, (1974).

Reagents

1. Sodium phosphate buffer (0.01M, pH7.0)

Disodium hydrogen phosphate (Na_2HPO_4) - 0.2 g and Sodium dihydrogen phosphate (NaH_2PO_4) - 0.05 g dissolved in 100 ml distilled water.

2. H_2O_2 (30% W/V)

Protocol

0.1ml of the enzyme extract was added to the reaction mixture comprising 3ml of H_2O_2 and 2.9 ml of 0.01M phosphate buffer (pH 7.0) and the OD change at 240 nm was measured over 10 minutes. The enzyme activity is measured in µmole of H_2O_2 consumed/min/mg protein. UV-Spectrophotometer (Systronics-AU-2701) was used to measure the specific activity of the enzyme.

$$Specific activity \mu(moles/min)/mg protein = \frac{\Delta A \times TV \times 1000}{K \times V of sample \times mg protein}$$

ΔA= Change in absorbance [$(F_A-I_A) / (F_T-I_T)$], TV= Total volume

K= Extinction coefficient ($6.93\times10^{-3}mM^{-1}cm^{-1}$), V= Volume of sample

3.7.4. Superoxide Dismutase Activity (SOD)

Activity of superoxide dismutase was done by using the procedure of Marklund and Marklund (1974).

Reagents

1. Tris EDTA Buffer (pH8.2)

2.85 g of Tris and 1.11 g of Ethylene diaminetetraacetic acid disodium ($EDTA-Na_2$) were mixed with 1L distilled water.

2. Pyrogallol (0.2mM) $C_6H_3 (OH_3)$

0.6 ml of concentrated hydrochloric acid was made up to 1 litter with distilled water and added 0.252 g of pyrogallol to make up the solution.

Procedure

50 µl sample and 1000 µl tris buffer were taken in a test tube against Tris EDTA buffer as blank. Using this blank spectrophotometer (Systronics AU-2701) was adjusted to zero and absorbance measured at 420 nm after one minute of the addition of pyrogallol.

Calculation of SOD Activity

$$\% \, Inhibion \, of \, pyrogallol \, autoxidation = \frac{A \, test}{A \, control} \times 100$$

$$(Cu-Zn) SOD \, Activity (U/ml) = \frac{\% \, Inhibition \, of \, pyrogallol \, autoxidation}{50\%}$$

3.7.5. Peroxidase Assay (POX)

The method of Reddy *et al*., (1995) used to assay the activity of peroxidase enzyme.

Reagents

1. 0.1M Sodium phosphate buffer (pH 6.5)

2. 0.005 M Catechol

3. 5mM Hydrogen peroxide

Protocol

Make up a reaction mixture with 2.5 ml of sodium phosphate buffer, 0.25 ml of catechol, 0.20 ml of H_2O_2 and 50µl of crude extract in a total volume of 3.0 ml at room temperature. The absorbance change was measured at 420 nm for 5 minutes. The enzyme activity is expressed as µmoles/min/mg protein.

$$Specific\ activity\ \mu moles/min/mg\ protein = \frac{(\Delta A \times\ T\,V \times\ 1000)}{(K \times V\ of\ sample \times mg\ protein)}$$

ΔA= Change in absorbance [$(F_A$-$I_A)$ / $(F_T$-$I_T)$], TV= Total volume

K for catechol oxidase = 0.272, V= Volume of sample

3.7.6. Assay of Glutathione S-Transferase (GST)

Determination of activity of glutathione S-transferase was done by adopting the method of Habig *et al.,* (1974).

Reagents

1. 100mM Phosphate buffer (pH6.5)

2. Reduced glutathione 50mM in phosphate buffer

3. 50 mM1-Chloro-2,4-dinitrobenzene (CDNB) in ethanol

4. Enzyme stock:10,000 rpm supernatant of tissue homogenate

5. 100 mM sodium phosphate buffer (pH 6.5), containing 1mM EDTA.

Protocol

1. 2.77 ml Phosphate buffer (100mM, pH 6.5) taken in a test tube and mixed with 50 µl of 50 mM CDNB and150 µl 50 mM reduced glutathione.
2. Then add 30 µl of enzyme stock to the above mixture.
3. Gently shake the mixture and incubate for 2-3 minutes at 25ºC.
4. Sample placed in the sample slot and reaction mixture without enzyme placed in the reference slot of the spectrophotometer (Systronics AU-2701). Follow the change in absorbance for three minutes at 340 nm (ε_{340}= 9.6 mM^{-1} cm^{-1}).

$CDNB - GSH\ conjugate$ f$ormed\ in\ \mu moles$ $min^{-1} mg^{-1}$ protein

$$= \frac{\text{ABS (increase in 3 min)} \times \text{TV} \times 1000}{\text{K} \times \text{V of sample} \times \text{mg protein}}$$

TV = Total volume, K=9.6 $mM^{-1}cm^{-1}$, V=Volume of sample

3.7.7. Assay of Glutathione peroxidase (GPx)

The method of Rotruck *et al.*, (1973) adopted to find out the activity of glutathione peroxidase enzyme. GPx activity was expressed in µmol GSH oxidized/min/mg protein.

Reagents

1. 0.4M Phosphate buffer (pH7.0)
2. 4mM Sodium Azide
3. 4 Mm Reduced glutathione
4. 2mM Hydrogen peroxide (freshly prepared from 30%)
5. 10% TCA
6. 0.3M Disodium Hydrogen Phosphate (Na_2HPO_4)
7. Elman’s reagent [DTNB-5,5’-Dithiobis(2-nitrobenzoic acid)]
8. Standard reduced glutathione

Protocol

1. Add 0.5ml buffer, 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H_2O_2 and 0.1 ml homogenate and 2 ml distilled water.
2. The sample tubes were incubated at 37° C for 3 minutes and after the incubation period, the reaction was terminated by the addition of 0.5ml TCA.
3. After centrifugation at 3000 RPM, the supernatant was collected to check the residual glutathione content by adding 4 ml of Na_2HPO_4 and 1 ml DTNB reagent.
4. The reaction produces a yellow color. The absorbance was read against reagent blank containing phosphate solution and DTNB at 412 nm.

$$Specific\ activity(\ \mu moles\ /\ mg\ protein) = \frac{OD\ of\ Test \times Conc.of\ standard \times TV \times 1000}{OD\ of\ Standard \times Volume\ of\ enzyme \times mg\ protein}$$

3.7.8. Glutathione Reductase (GR)

The glutathione reductase activity was done by using the method of David and Richard (1983).

Reagents

1. 0.12 M Phosphate buffer (pH 7.2)
2. 1mM EDTA
3. 50 mM Sodium azide
4. 50 mM Oxidized glutathione
5. 4 mM NADPH

Protocol

To 0.1 ml sample, 1ml phosphate buffer (0.12 M pH 7.2), 0.1 ml of EDTA, 0.1 ml sodium Azide and 0.1ml oxidized glutathione were added and the volume was made up to 2 ml with distilled water. The mixture was kept at room temperature for 3 minutes and 0.1 ml NADPH added. The absorbance read at 340 nm for 5 min. One unit of GR is expressed as µM of NADPH oxidized/minute/ mg protein.

$$Specific\ activity(\mu moles/mg\ protein) = \frac{\Delta A \times TV \times 1000}{6.22 \times V\ of\ sample \times mg\ protein}$$

3.8. Molecular parameters

Molecular experiments conducted in various developmental stages such as larva, pupa and adult. Silk gland, midgut, fat body and haemolymph were collected from larval stage, fat body of pupa and adult, haemolymph of pupae were also used for the molecular analysis. Five larvae were randomly selected from the control and experimental groups were dissected out and collected in pre frozen vials filled with Trizol reagent and stored at -20 ºC (Low temperature freezer Labline –VDF-2851-S).

3.8.1. mRNA Isolation

Total RNA were extracted from the tissues like silk gland, mid gut and fat bodies of larva, fat body of pupa and adult, haemolymph of larvae and pupae using Aurum total RNA extraction kit (Bio Rad) followed by manufacturer's directions as mentioned below.

1. 100 mg of tissue samples were accurately weighed and homogenized in liquid nitrogen using a mortar and pestle. 100 µl of haemolymph samples were also mixed with liquid nitrogen. This process helps to increase the cell surface area exposed to the lysis buffer while simultaneously inhibiting ribonucleases. The sample was not allowed to defrost in between grinding steps.
2. Following the lysis buffer, the lysate frequently becomes highly viscous due to the release of genomic DNA into the solution. It is critical to lower the viscosity of lysate using the REMI Lab stirrer homogenizer.
3. Centrifugation carried out for 3 minutes and transferred the supernatant to a fresh 2 ml capped micro centrifuge tube.
4. The resulting suspension was transferred to the RNA binding column provided with the kit and centrifuged for 60 sec at 13,000 RPM.
5. 700µl of low stringency solution was used to rinse the RNA binding column and centrifugation carried out at 13,000 rpm for 30 seconds.
6. Low stringency wash solution was discarded from the wash tube.

7. It added 80 µl of the given diluted DNAse I to the membrane stack at the bottom of each column.
8. Expulsion of genomic DNA done by incubation of digest at room temperature for 25 minutes.
9. The RNA binding column was washed twice, first with high and second with low concentration of 700 µl stringency washes solution.
10. Following the washes, the column was centrifuged for two minutes to get rid of the remaining wash solution.
11. Then the RNA binding column was transferred to a fresh 1.5 ml capped micro centrifuge tube and added 80 µl of the eluted solution on to the membrane stack at the bottom of the RNA binding column.
12. This was left without any interruption for one minute to allow the solution to saturate the membrane.
13. Following the incubation, the sample was centrifuged for two minutes to elute the total RNA.
14. RNA quality was tested using agarose gel electrophoresis (Biotech Himedia) by loading 2µl of the sample.
15. The retrieved RNA was quantified at 260 nm, and purity was determined at 260/280 nm.

3.8.2. Synthesis of cDNA

Isolated total mRNA converted to c DNA by using 2x RT Easy Mix supplied by G Bioscience. The reaction was carried out by incubating the reaction mixture in a thermal cycler (CFX connect Real Time System- Bio Rad) according to the instructions protocol.

Table 3.1. Composition of reaction mixture

Component	Volume per reaction µl
2XRTEasy Mix	5 µl
Random primer (50µM)/ Oligo (Dt)18 primer (50µM)	0.5µl
RNA template	1 µl
RNAse free ddH_2O	3.5 µl
Total volume	10µl

3.8.3. Reaction protocol

Incubate the reaction mixture in a thermal cycler (CFX connect Real Time System - Bio Rad) using the following protocol.

Table 3.2. Reaction protocol

Reverse transcription	20 minutes at 42ºC
RT inactivation	5 minutes at 85ºC

The ensuing cDNA was conserved for upcoming applications in PCR or RT. As per the manufacturer's instructions, RNA was extracted from the samples using Aurum total RNA mini kit (Biorad).5µl of each obtained RNA samples were loaded into 1% agarose gel, then the image was captured with the Gelstan 4X advanced software and the Gel i ink doc system.

3.8.4. qRTPCR analysis

The PCR reaction were carried out in 10 µl reaction mixture containing 5 µl 2X SYBR Green qPCR mix(G Bioscience), 1µl of 10µM forward and reverse primer, 1µl of template DNA and 2 µl Nuclease free water. *Bombyx mori* Actin gene (BmActin) was used as the reference gene. The gene expression was analyzed using the $2^{\wedge-}\Delta\Delta CT$ method proposed by Livak and Schmittgen (2001).

Table 3.3. Real time PCR Mix

PCR Components	Stock	Volume to be taken
Nuclease free water	-	2 µl
Forward Primer	10 µM	1 µl
Reverse Primer	10 µM	1µl
SYBR Green qPCR Mix	2X	5µl
Template Cdna	-	1 µl
Total Volume		10 µl

The amplification reactions were performed in a CFX connect Real Time System (Bio Rad). Following PCR conditions with the steps of initial denaturation at 95ºC for 10 minutes followed by 40 cycles of 95ºC for 10 secs , and 72ºC for 40 secs. The annealing temperatures of all the genes for the current primers were calculated using the Tm of the forward and reverse primers.

Table 3.4. PCR Conditions

Profile	Temperature	Time
Initial denaturation	95ºC	00:10:00
Denaturation	95ºC	00:00:10
Annealing	BmActin 56ºC HSP70 58ºC Caspase-3 52ºC BmYki 52ºC MBF1 65ºC BmApaf-1 52ºC BmICE-2 57ºC IL6 58ºC TNFSF5 60ºC	00:00:40
Extension	72ºC	00:00:50

3.8.5. List of primers

The following primers were used for mRNA expression analysis.

Table 3.5. Primer list

Genes		Primer sequences (5'-3')	Reference/ cDNA accession number
BmActin	FP	CGGGAAATCGTTCGTGAT	Li *et al.*,(2010)
	RP	ACGAGGGTTGGAAGAGGG	
BmYki	FP	CGAAGAGTACAAGTAATACGACAA	Zeng *et al.,*(2017)
	RP	TACGAGCTGCGTGATTAATG	
MBF1	FP	AAGGATCCATGTCTGACTGGGATACAGT	Li *et al.*,(2009)
	RP	ACAAAGCTTTTATTTCTGTCCGCCAGGAG	
BmApaf-1	FP	GGTTTGCTCGTAATGGAC	Zhang *et al.,* (2010)
	RP	ATGCGTTGGAAGGCGTAA	
BmICE-2	FP	GGCGATAGCGGCGAAGTA	Franzetti *et al.*,(2012)
	RP	ATGCGTTGGAAGGCGTAA	
IL6	FP	TCATGGCTCAGTTTCCGACC	Fang *et al.*,(2022)
	RP	AGTCGTCAAAGGTCAGCTCG	
TNFSF5	FP	GCTCTGGTTGCCCCAAAATC	Fang *et al.*,(2022)
	RP	GGACCCATTAATGCTTTTTGTAGGA	
HSP70	FP	GAACACACTCGCTGCACATC	DQ311189.1
	RP	GAGGAGTGCCCAAGATCCAC	
Caspase-3	FP	AGTTTCGGTCATCTGCTTTAC	Hu *et al.*, (2018)
	RP	CATTCGGACTTCCTCTTCTTA	

3.9. Histopathology of midgut

The fifth instar larval silkworm tissue sample midgut was dissected out from each group and washed with normal saline solution then it instantaneously fixed in Bouin's fluid. Following fixation, tissue sample dehydrated with series of ethanol using 30%, 50%, 70%, 85%, 95% and 100%, each step lasting for 15 minutes. Then the sample was treated twice with xylene for again 15 minutes then processed for embedding in paraffin wax and sectioned using a microtome (Leica RM 2125 RT). The sections were cut with five micron thickness and dewaxed using xylene for 15 minutes. Rehydration is achieved by passing the section through descending series of alcohol. Tissue sections placed on a glass slide and the sections were stained with haematoxylin and eosin (Humason, 1962; Chavan *et al*., 2015). Photographs were taken by using a light microscope (Leica ICC 50 E).

3.10. Quality and quantity parameters of cocoon

All the quality and quantity parameters of cocoon were assessed in according to the standard protocol (FAO manual).

3.10.1. Average weight of single cocoon: Ten cocoons were randomly selected from control and test group on 6th day of spinning and weighed using electronic balance (SCALE TEC-SAB203) and reckoned the average.

3.10.2. Percentage of good cocoons: Damaged, unpinned and unsized cocoons from each group were removed and rate of good cocoons were calculated by the following formula.

$$Percentage\ of\ good\ cocoons = \frac{Good\ cocoon\ (reelable\ cocoon)}{Total\ number\ of\ cocoons} \times 100$$

3.10.3. Shell weight of cocoon: Prior to weighing the shell weight, ten cocoons were randomly selected from each group and removed the pupae then calculated the average weight.

3.10.4. Shell ratio: Shell ratio was measured using the following formula accordingly.

$$Shell\,ratio = \frac{Shell\,weight}{Cocoon\,weight} \text{ x } 100$$

3.10.5. Filament length: Length of silk filament indicates the reelable length of silk filament from a cocoon. It is measured by using an Epprovate machine and the length recorded accordingly in meters.

3.10.6. Filament size or Denier of the cocoon: Denier of the cocoon calculated by the following formula.

$$Denier = \frac{Filament\,weight}{Length\,of\,filament} \text{ x } 9000$$

3.10.7. Reelability Percentage: It indicates the percentage of reeling ability of a cocoon.

$$Reelability\,percentage = \frac{weight\,of\,the\,cocoon\,filament}{weight\,of\,the\,cocoon} \text{ x } 100$$

3.11. Statistical analysis

Statistical analysis of the collected data was done by using Origin statistical software (version 2023) and Microsoft Excel 2007. One way analysis of Variance (ANOVA) was conducted, followed by mean comparisons using Tukey's test. Results are presented as mean ± standard deviation. Biochemical, morphometric and cocoon parameters were analyzed using six replicates. Means that do not share a common letter are considered significantly different. Letters (eg. 'a' and 'b') shown in figures indicate significant differences among groups. Values sharing same letters are not significantly different. The group denoted with letter 'a' mean is significantly higher than group denoted with letter 'b'. Molecular analysis was performed with three replicates and significance was indicated using an asterisk (*). Differences were considered statistically significant at $P<0.05$.

RESULTS

4. RESULTS

4.1. Morphometric parameters of silkworm

4.1.1. Total body weight of fifth instar larvae

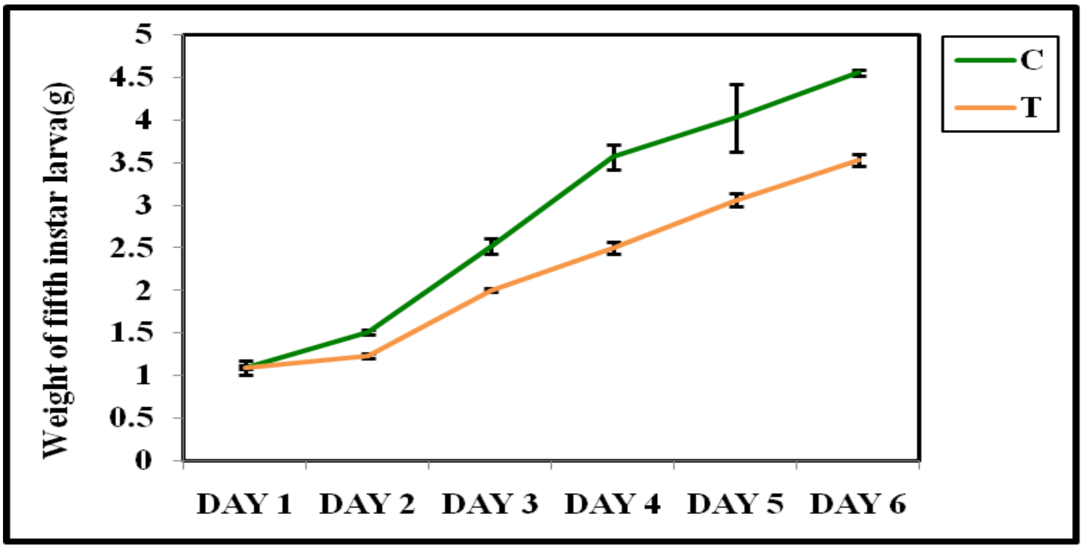
The periodical evaluation of the average body weight of fifth instar larvae between the control group and treated group, found a striking decline of weight in each day in the test group (T). Table 4.1 represents the periodical average weight gain of fifth instar larvae of silkworm during days one to six. Significant difference calculated by using ANOVA followed by Tukey's test.

Table 4.1: The average body weight (g) of fifth instar larvae exposed to thermal stress

DAYS	C	T
	CONTROL	THERMAL STRESS
DAY1	1.09± 0.08	1.09± 0.02
DAY2	1.51± 0.03	1.23± 0.03
DAY3	2.51± 0.09	2.0 ± 0.02
DAY4	3.56±0.14	2.5± 0.07
DAY5	4.02± 0.4	3.06± 0.07
DAY6	4.55± 0.04	3.53±0.07
Average weight of fifth instar larva	2.87 ± 0.13^{a}	2.24 ± 0.22^{b}

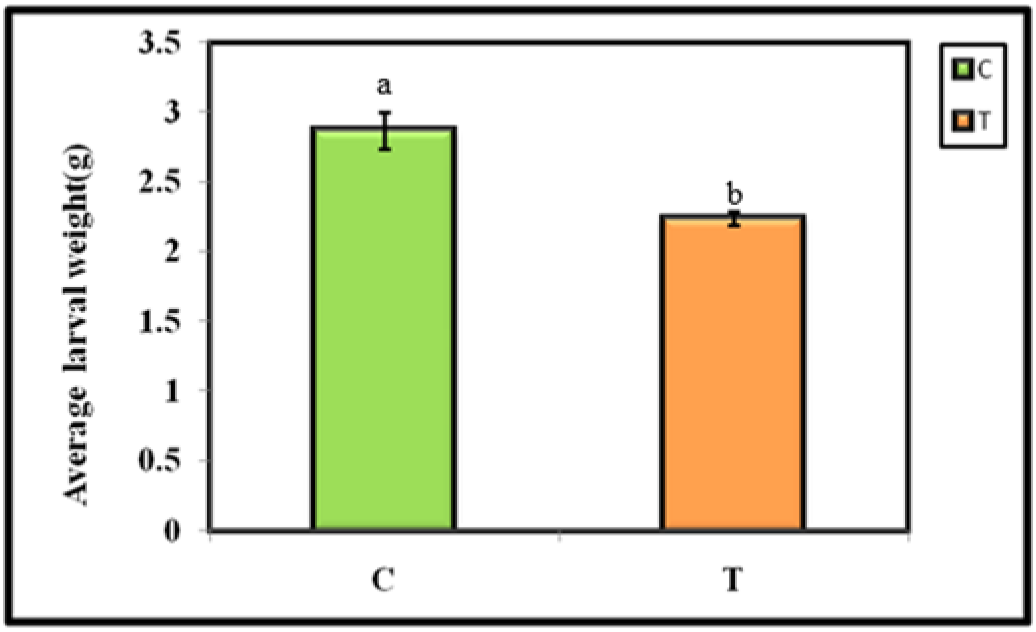
Means having the different superscript letters differ significantly at $P<0.05$ in ANOVA (Tukey's test). Data represented as mean± SD, n=6.

Figure 4.1: Periodical body weight gain of fifth instar larvae exposed to temperature stress



Note: C- Control, T- Thermal stress

Figure 4.2. Average bodyweight of fifth instar larvae exposed to temperature stress



Note: C- Control, T- Thermal stress

4.1.2. Average body weight of pupa and adult

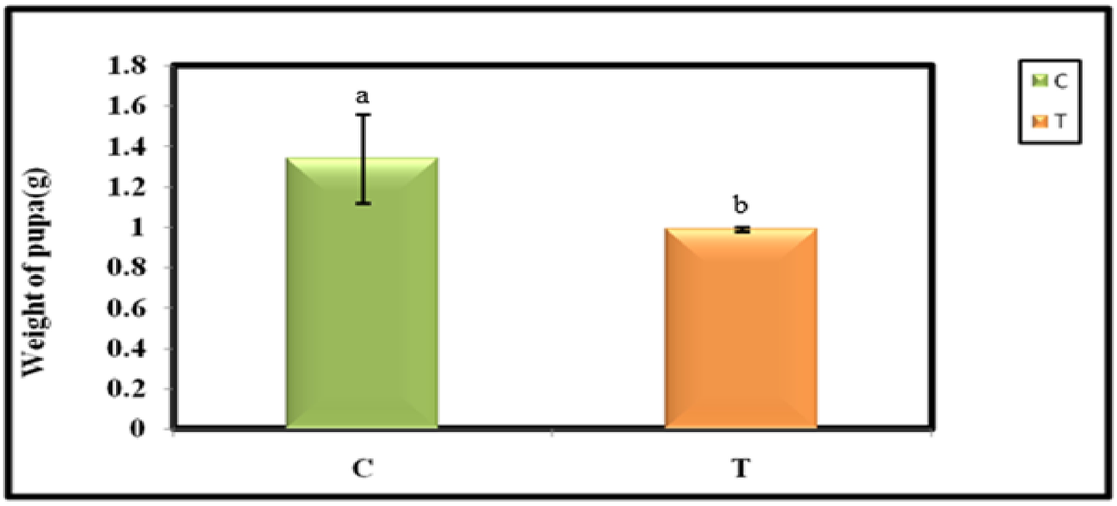
The average body weight of pupae and adults periodically measured on exposure to thermal stress showed a marked decline in the treatment group with respect to control group. A significantly lower pupal weight was noticed in the experimental batch relative to control. The gender-specific body weight was measured in adults. The mean weight of the control pupae and test pupae were 1.34 ± 0.02 & 0.99 ± 0.009 correspondingly. The mean body weight of the control and experimental group of an adult male was 0.31±0.072& 0.17 ± 0.032 and the female was 0.72± 0.2 &0.39± 0.073 respectively. The female adult showed a substantial decline ($P<0.05$) in the treated group benchmarked against control.

Table 4.2: Average body weight of pupa and adult in response to thermal stress

DEVELOPMENTAL STAGE	C	T
	CONTROL	THERMAL STRESS
Pupa	1.34±0.02[a]	0.99±0.009[b]
Adult(Male)	0.31±0.072[a]	0.17±0.032[b]
Adult(Female)	0.72±0.2[a]	0.39±0.073[b]

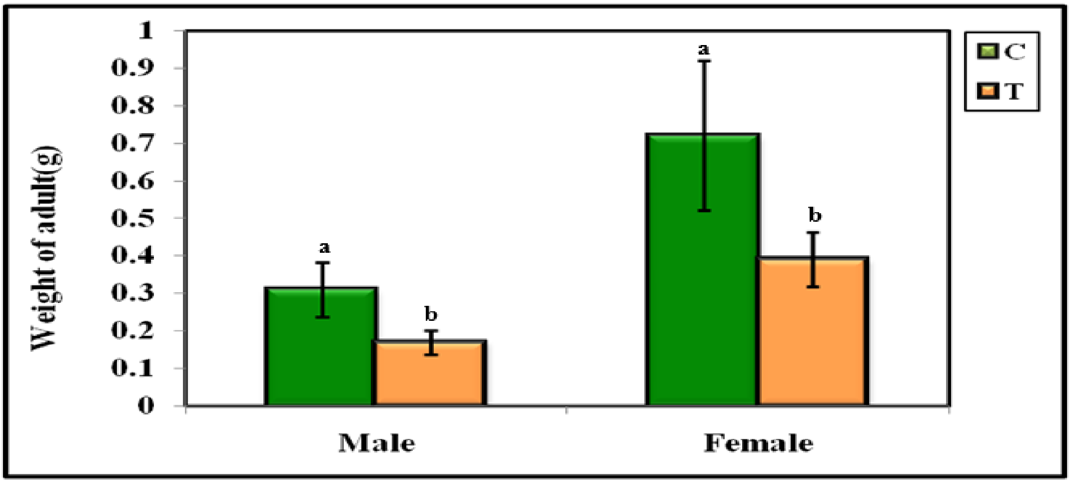
Each value represents the mean of (±SD) 6 replications. Different letters in each column indicates significant differences at $P<0.05$ in ANOVA (Tukey's test).

Figure 4.3: Average body weight of pupa of control and thermal stress exposed group



Note : C- Control, T- Thermal stress

Figure 4.4: Average body weight of male and female adults in response to thermal stress



Note: C- Control, T- Thermal stress

4.1.3. Average body length of fifth instar larvae

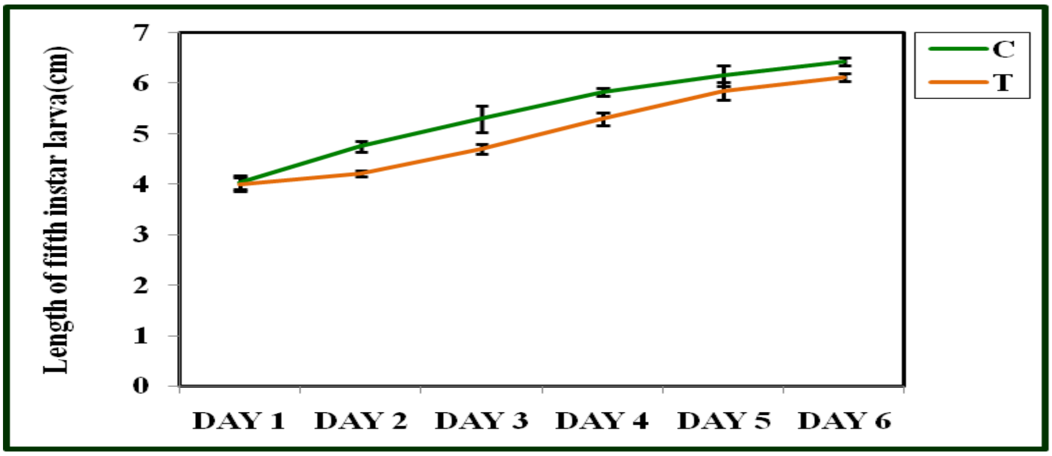
Throughout the experimental period, the periodical measurement of the entire body length of the fifth instar larvae revealed a substantial decline in thermal stress exposed group compared to control. The control group showed the largest average body length. Table 4.3 depicts the periodical measurements of fifth phase larval length over a six day period, comparing both the control and experimental groups.

Table 4.3: Mean body length of silkworm fifth instar larvae of control and treated group

DAYS	C	T
	CONTROL	THERMAL STRESS
DAY1	4.03±0.136	4.0 ± 0.14
DAY2	4.75 ± 0.105	4.22 ± 0.08
DAY3	5.3±0.261	4.7± 0.09
DAY4	5.83±0.082	5.3±0.13
DAY5	6.15±0.2	5.85±0.18
DAY6	6.43± 0.082	6.12± 0.08
Average length of fifth instar silkworm larvae	5.415 ± 0.144^{a}	5.031 ± 0.113^{b}

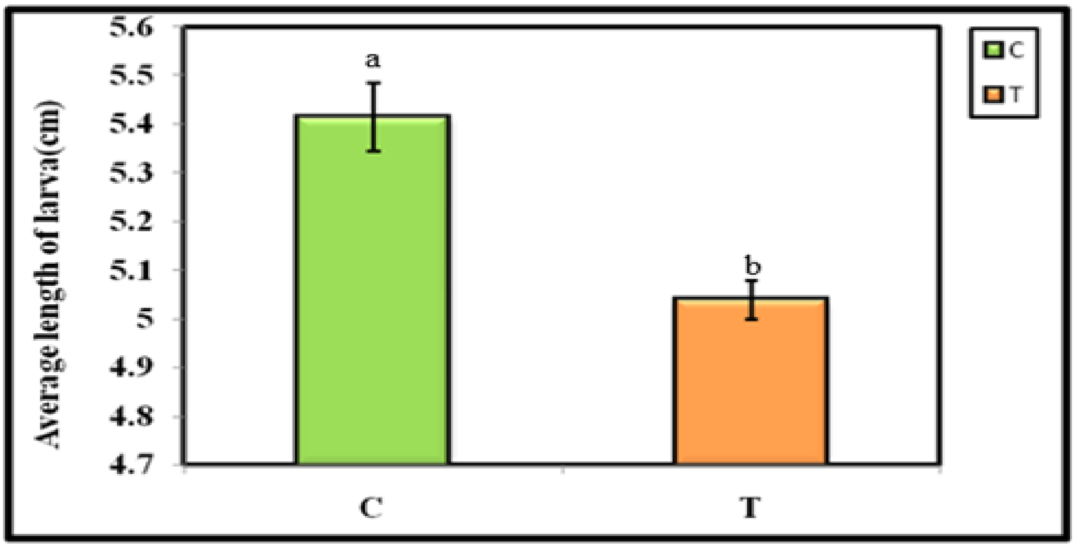
Means having the different superscript letters differ significantly at P<0.05 in ANOVA (Tukey's test). Data represented as mean± SD, n=6.

Figure 4.5: Body length gain of fifth instar larvae of control and treated group



Note: C- Control, T- Thermal stress

Figure 4.6: Average body length of fifth instar larvae of control and experimental group



Note : C- Control, T- Thermal stress

4.2. Variation in biological characters

4.2.1. Mortality percentage

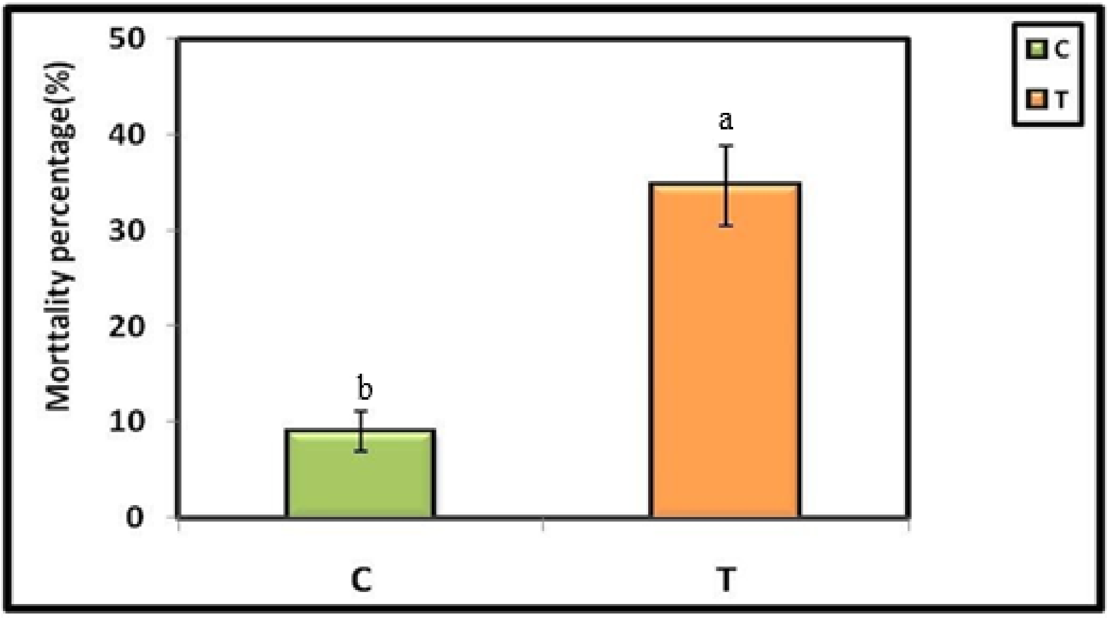
The mortality percentage of the two groups was calculated and the results showed that thermal stress-exposed experimental groups had a significantly ($P<0.05$) higher mortality percentage (34.66±4.13) compared to control (9±2.09).

Table 4.4: The average mortality rate of fifth instar silkworm larvae underwent heat shock

PARAMETER	C	T
Mortality rate	CONTROL	THERMAL STRESS
	9 ± 2.09^{b}	34.66 ± 4.13^{a}

The significance of difference is indicated as by different superscript letters (P<0.05, ANOVA, Tukey's test). The values are presented as mean ± SD, n=6.

Figure 4.7. The average mortality rate of fifth instar larvae of control and experimental group



Note: C- Control, T- Thermal stress

4.3. Biochemical parameters

4.3.1. Measurement of lipid peroxidation level (MDA content)

Changes in the lipid peroxidation level were detected in the various tissues of larval, pupal and adult stages of silkworm. All the selected tissues such as the silk gland (30.41±0.69) and midgut (19.06±0.41) of the larva, fat body of larva (12.17±0.61), pupa (0.7±0.036) and adult (1.15±0.108) of thermal stress exposed group showed a significant (P<0.05) increase in the lipid peroxidation level compared to control.

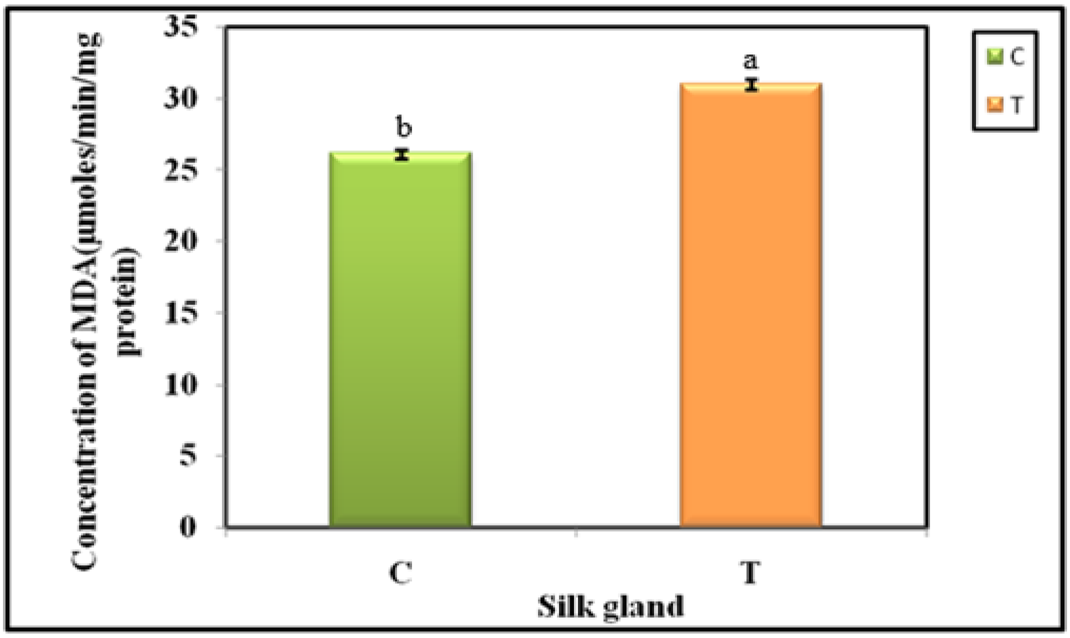
Table 4.5. Level of lipid peroxidation in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	25.43±0.45[b]	30.41±0.69[a]
Midgut	14.33±0.58[b]	19.06±0.41[a]
Fat body	8.15±0.65[b]	12.17±0.61[a]
Pupal fat body	0.16±0.017[b]	0.7±0.036[a]
Adult fat body	0.9±0.073[b]	1.15±0.108[a]

Means that do not share a letter are significantly different at P<0.05 in ANOVA (Tukey's test). The data represented as mean ±SD, n=6.

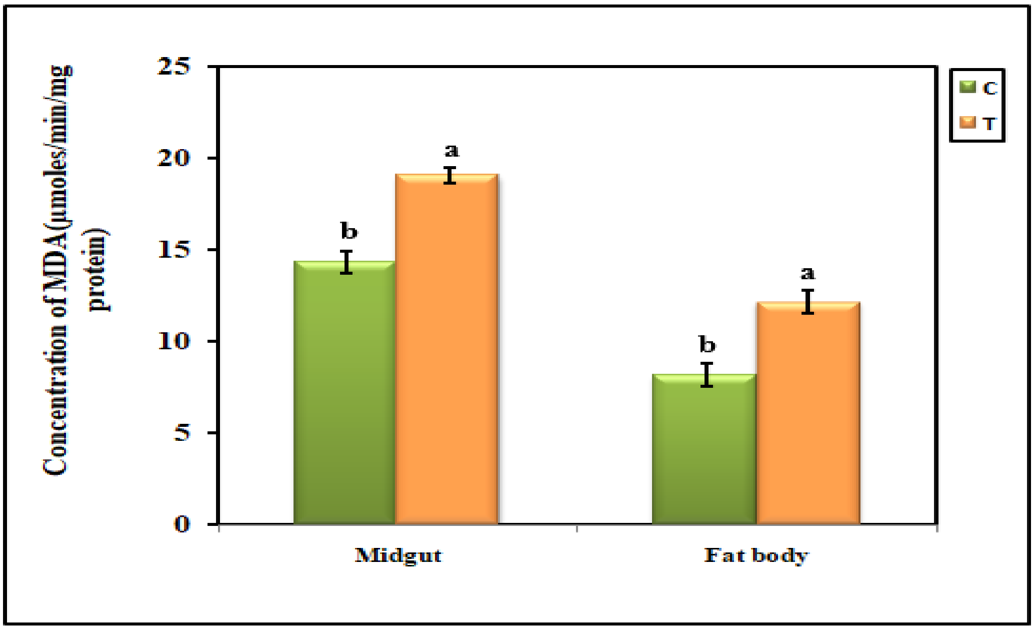
Figure 4.8. Changes in lipid peroxidation levels in the larval pupal and adult tissues of the control and treated group

(a) Alterations in lipid peroxidation level in the larval silk gland of control and thermal stress exposed group



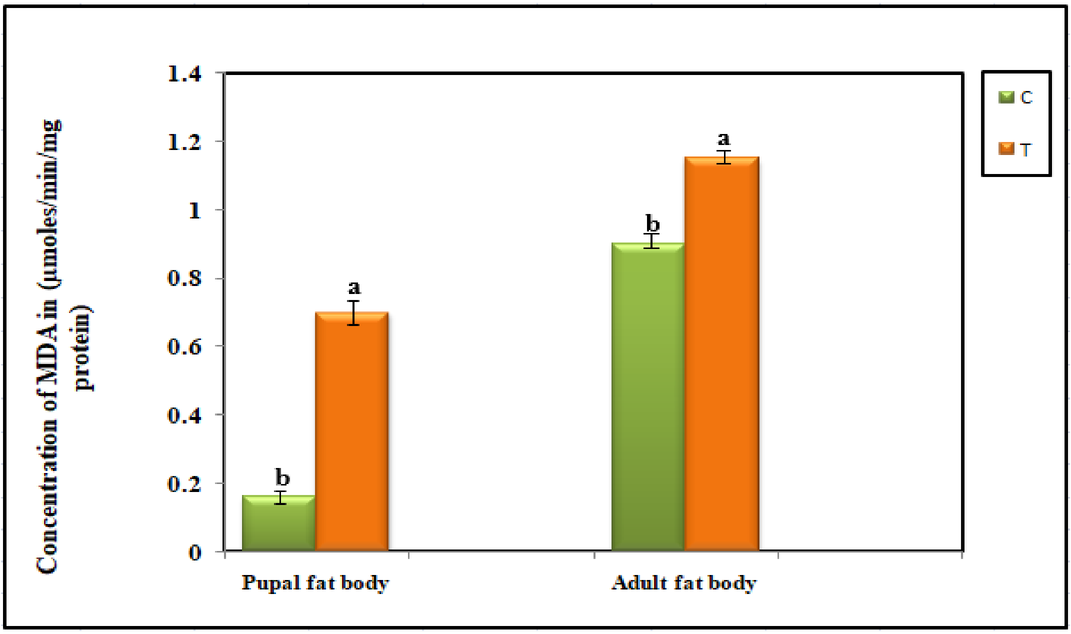
Note : C- Control, T- Thermal stress

(b) Alterations in lipid peroxidation level in the larval midgut and fat body of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

(c). Alterations in lipid peroxidation level in the pupal and adult fat body of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

4.3.2. Total protein concentration

The total protein concentration significantly varied among different life stages of silkworms in response to thermal stress. The silk gland (2.2±0.08[a]) and fat body (2.69±0.07[a]) of fifth instar larvae showed significantly increased total protein concentration in the thermal stress treated samples .While the midgut (1.652±0.071[b]) of the fifth instar larvae displayed a diminished total protein concentration compared to the control. The pupal (3.28±0.084[b]) and adult (0.55±0.03[b]) developmental stages of the fat body exhibited a substantial decrease in the total protein concentration in the test group measured against the control.

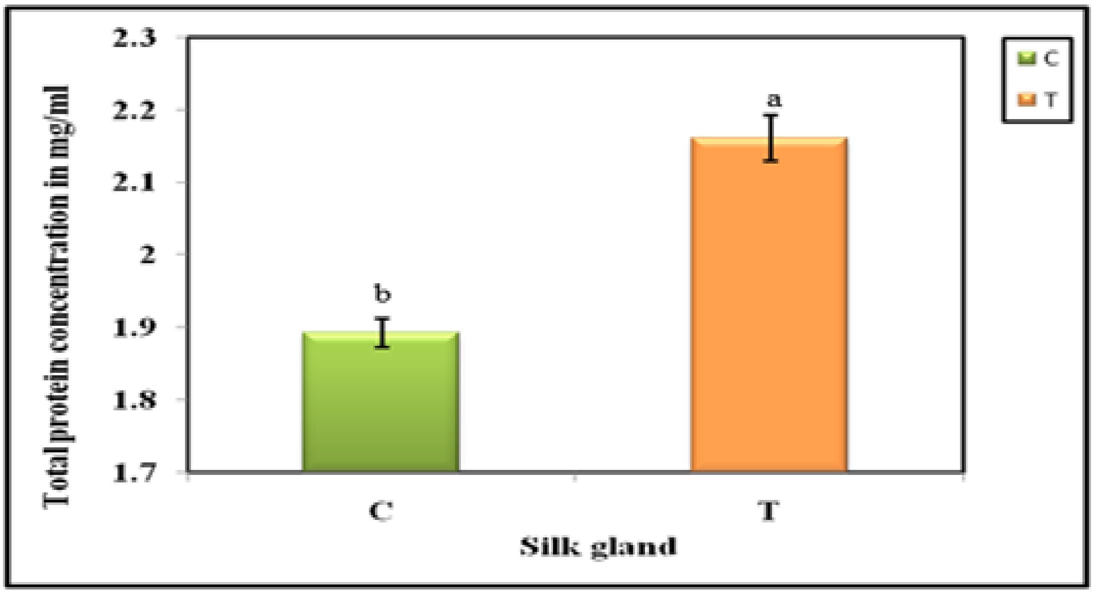
Table 4.6. Total protein concentration in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	25.43±0.45[b]	30.41±0.69[a]
Midgut	14.33±0.58[b]	19.06±0.41[a]
Fat body	8.15±0.65[b]	12.17±0.61[a]
Pupal fat body	0.16±0.017[b]	0.7±0.036[a]
Adult fat body	0.9±0.073[b]	1.15±0.108[a]

Means that do not share a letter are significantly different at $P<0.05$ in ANOVA (Tukey’s test). The data represented as mean ±SD, n=6.

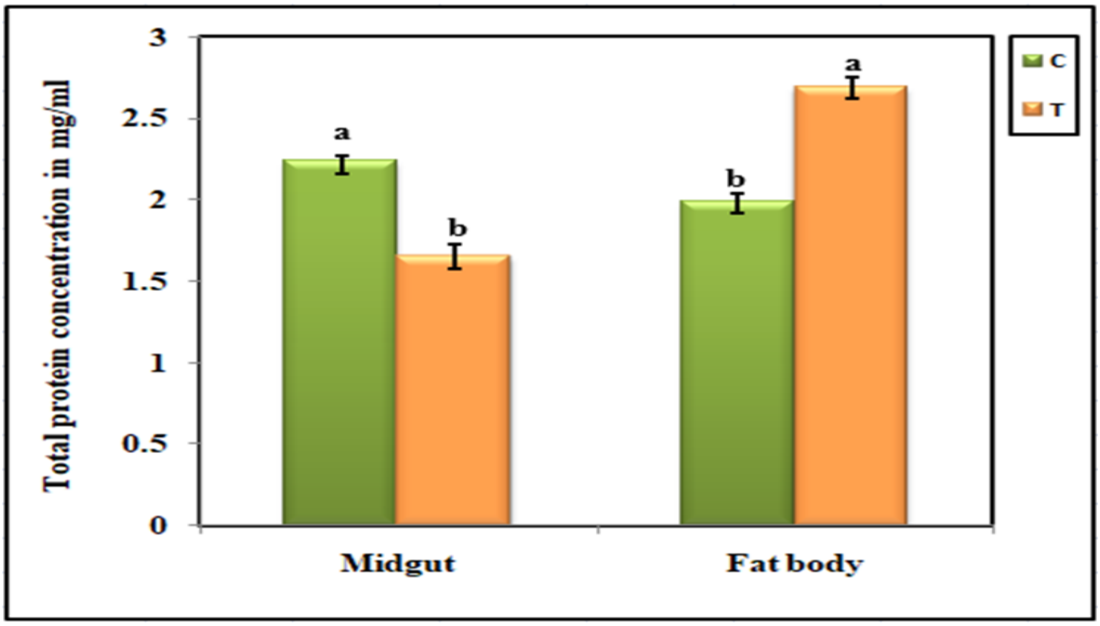
Figure 4.9. Total protein concentration in the larval pupal and adult tissues of control and treated group

(a) Total protein concentration in the larval silk gland of control and treated group



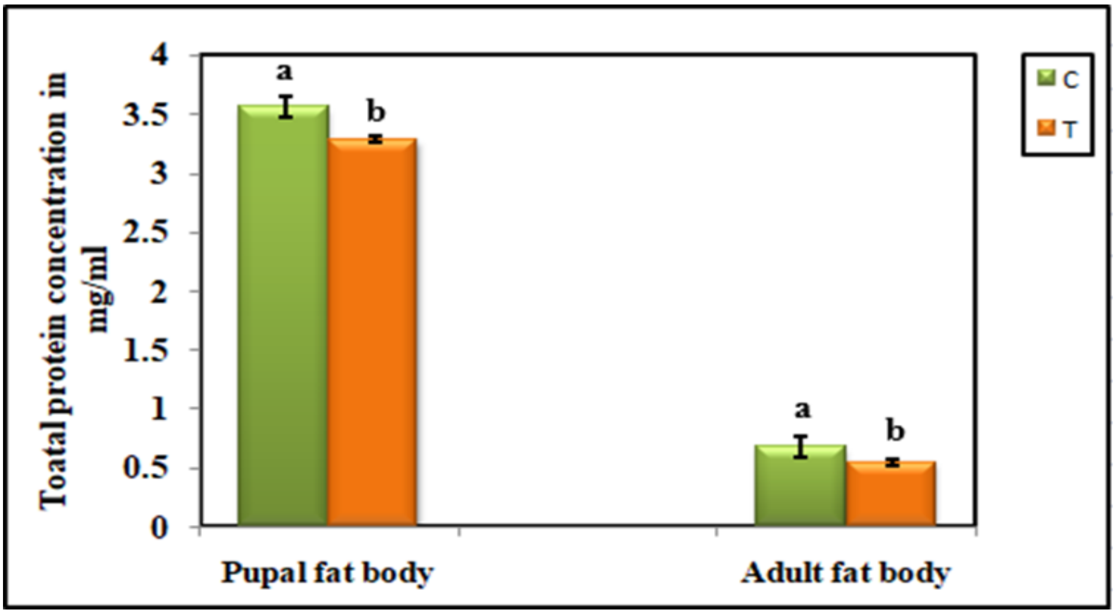
Note: C- Control, T- Thermal stress

(b) Total protein concentration in the larval midgut and fat body of control and treated group



Note: C- Control, T- Thermal stress

(c) Total protein concentration in the fat body of pupa and adult of control and treated group



Note: C- Control, T- Thermal stress

4.4. Assay of antioxidant enzymes

The impact of thermal stress on the anti oxidative system of silkworm *Bombyx mori* was analyzed at different developmental stages such as larva, pupa and adult. The activity of important antioxidant enzymes specifically CAT, SOD, POX, GST, GPx, and GR had been analyzed and recorded as follows. The significant variation is represented with different superscript letters at $P<0.05$ in ANOVA, mean comparisons were done by using Tukey's test.

4.4.1. Catalase enzyme activity (CAT)

The activity of the CAT enzyme revealed a considerable increase in the silk gland of fifth instar larvae under the thermal stress group in comparison to the control. On the contrary, the midgut (14.7±0.834 µmoles /min/mg protein) and fat body (41.26±0.75 µmoles/min/mg protein) of thermal stress exposed group implicated a decreased enzyme activity in comparison with the control group. The fat body of the pupal and adult stage showed a similar trend of enzyme activity.

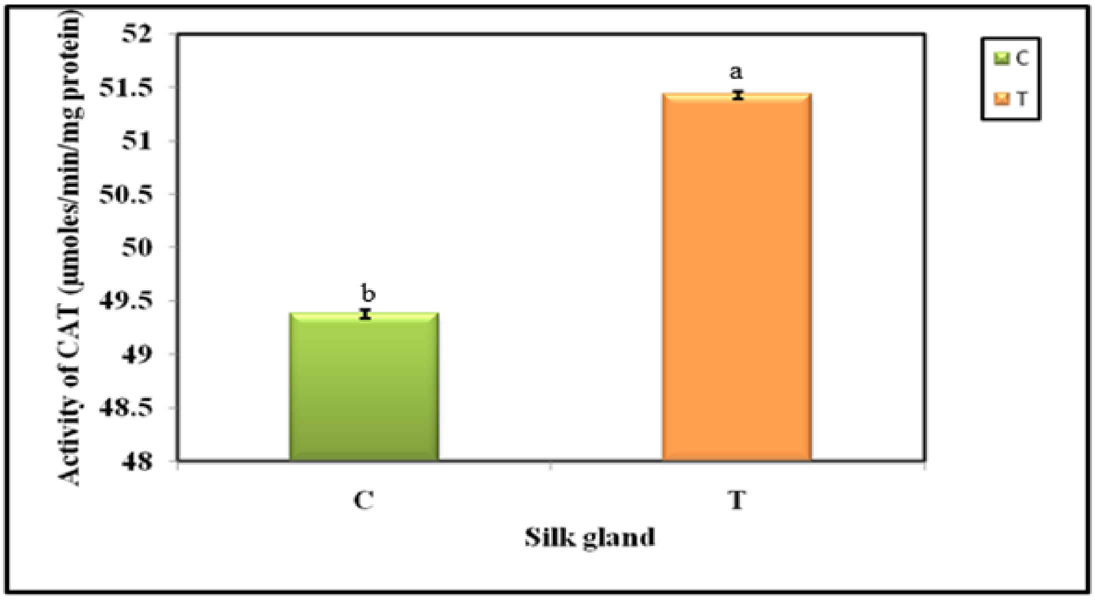
Table 4.7. Level of CAT enzyme activity in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	49.38±0.09[b]	51.44±0.085[a]
Midgut	18.2±0.14[a]	14.7±0.834[b]
Fat body	43.78±0.88[a]	41.26±0.75[b]
Pupal fat body	31.62±0.35[a]	29.17±0.2[b]
Adult fat body	23.42±0.7[a]	20.15±0.8[b]

The significance of difference is indicated as by different superscript letters (P<0.05, ANOVA, Tukey's test). The values are presented as mean ± SD, n=6.

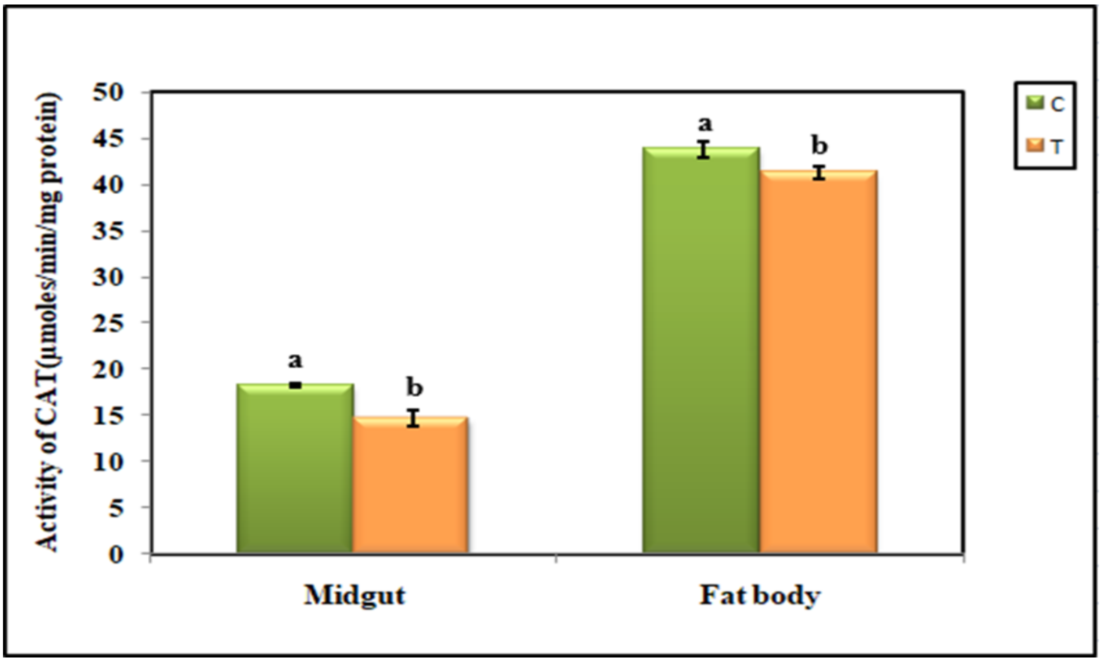
Figure 4.10. CAT enzyme activity in the larval pupal and adult tissues of control and treated group

(a) Activity of CAT in the silk gland of fifth instar larvae of control and treated group



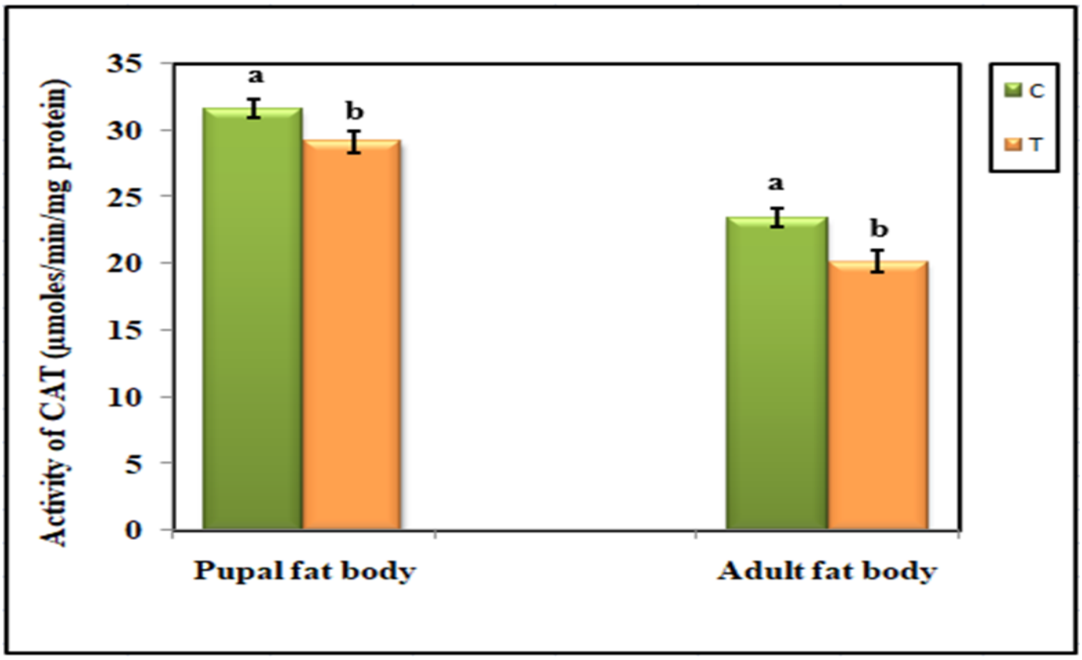
Note : C- Control, T- Thermal stress

(b). Activity of CAT in the midgut and fat body of fifth instar larvae of control and treated group



Note: C- Control, T- Thermal stress

(c) . Activity of CAT in the fat body of pupa and adult of control and treated group



Note: C- Control, T- Thermal stress

4.4.2. Superoxide dismutase activity (SOD)

SOD activity in the silk gland of the fifth instar larva was observed as 2.26±0.08[b] µmoles / min / mg protein in the untreated group while that of the experimental set is 2.44 ± 0.07[a] µmoles / min / mg protein. Silk gland SOD activity in the thermal stress treated worms was exceeded relative to the normal group. Exposure of silkworms to thermal stress significantly decreased the action SOD in the midgut and fat body of larvae, as in this experiment the SOD activity was 2.39±0.015[b] µmoles / min / mg protein and1.99 ± 0.07[a] µmoles / min/ mg protein correspondingly. The SOD activity in the fat body of the pupa of control (C), but that of the thermal stress exposed group (T) was 2.43±0.05[a], 2.37±0.02[a] respectively. The fat body of the adult control (2.17±0.06[a]) and thermal stress exposed group (1.83±0.08[b]) displayed variations in the action of SOD. The noted SOD activity in the fat body of adults showed a significant reduction in the experimental group.

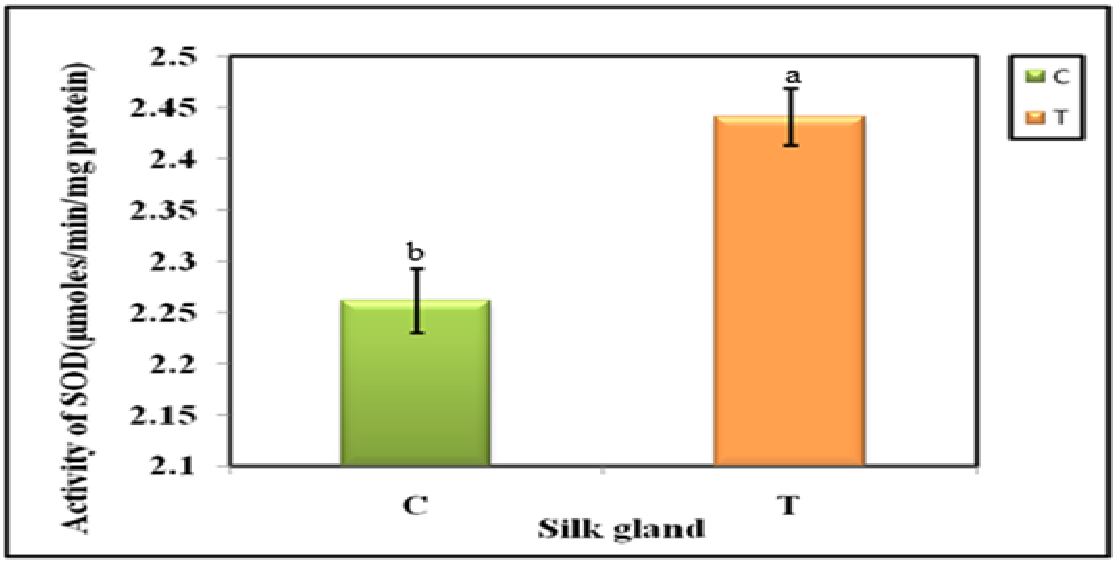
Table 4.8. Activity of SOD in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C CONTROL	T THERMAL STRESS
Silk gland	2.26±0.08[b]	2.44±0.07[a]
Midgut	2.67±0.18[a]	2.39±0.015[b]
Fat body	2.608±0.05[a]	1.99±0.07[b]
Pupal fat body	2.43±0.05[a]	2.37±0.02[a]
Adult fat body	2.17±0.06[a]	1.83±0.08[b]

The significance of difference is indicated as by different superscript letters ($P<0.05$, ANOVA, Tukey’s test). The values are presented as mean ± SD, n=6.

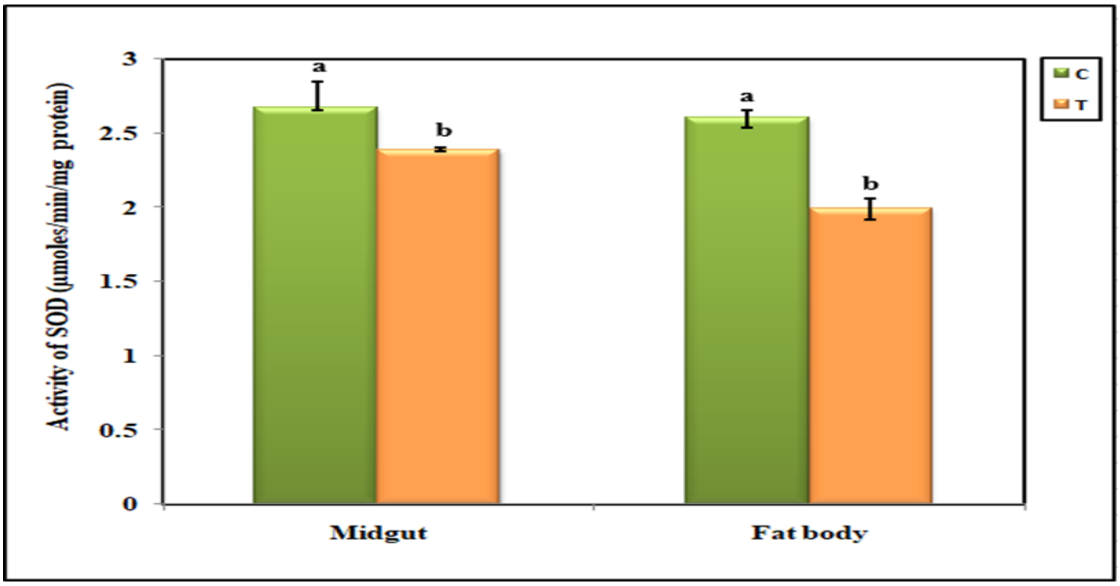
Figure 4.11. SOD activity in the larval pupal and adult tissues of control and treated group

(a). The activity of SOD in the silk gland of the fifth instar larvae of control and treated group



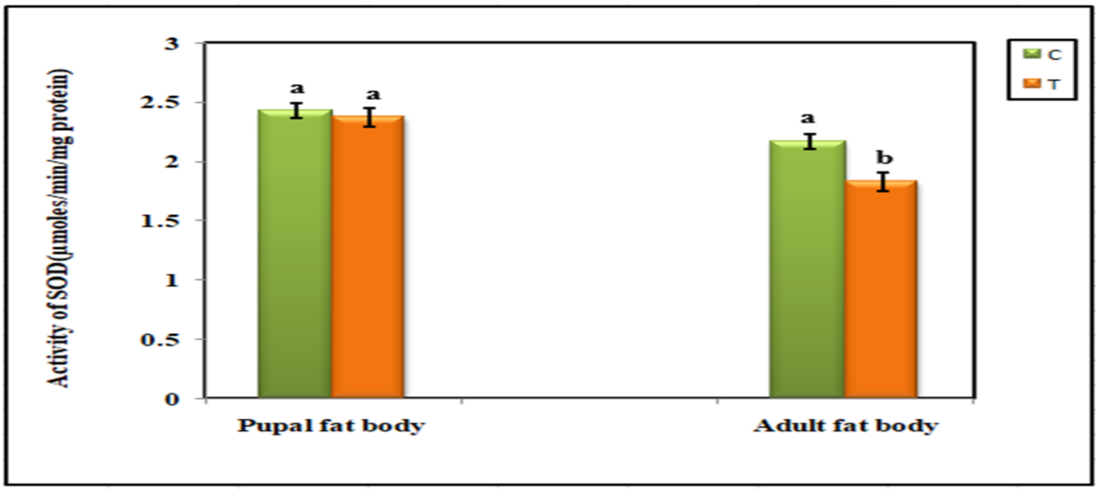
Note: C- Control, T- Thermal stress

(b). Activity of SOD in the midgut and fat body of fifth instar larvae of control and treated group



Note: C- Control, T- Thermal stress

(c). Activity of SOD in the fat body of pupa and adult of control and treated group



Note: C- Control, T- Thermal stress

4.4.3. Peroxidase enzyme activity

The activity of the POX enzyme was increased in the silk gland, midgut, and fat body of the fifth instar larva, and the fat body of pupa and adult. The POX activity significantly increased in the treated group of silk gland (119.3±0.08 µmoles/min/mg protein), while in the midgut (91.51±0.9108 µmoles /min/mg protein), and fat body (326.37±0.889108 µmoles /min/mg protein) of the treated group exhibited a significant decline in the activity of POX. Pupal and adult fat bodies also follow a similar trend.

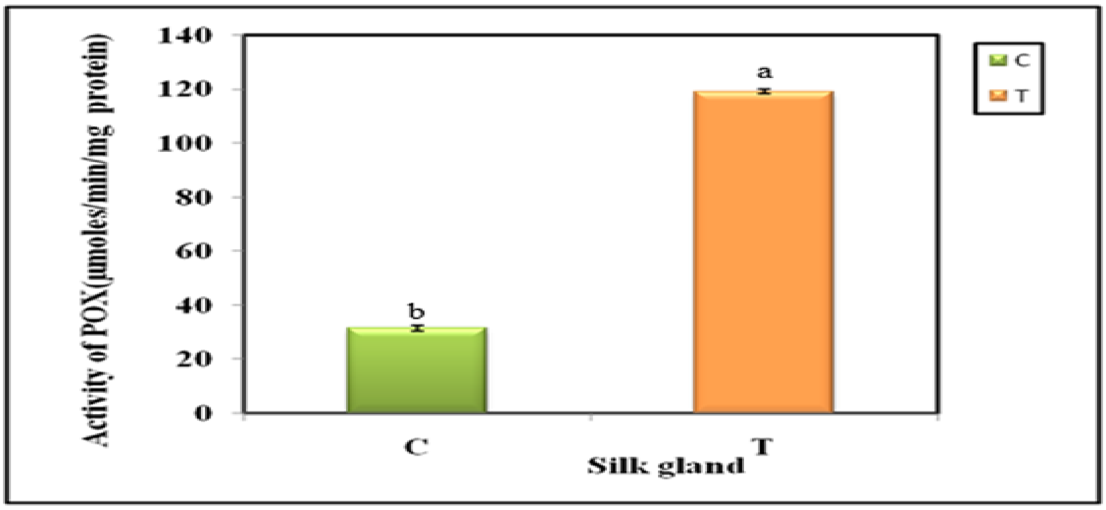
Table 4.9. The activity of POX in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	31.58 ± 0.08^{b}	119.3 ± 0.08^{a}
Midgut	129.70 ± 0.89^{a}	91.51 ± 0.91^{b}
Fat body	2511.6 ± 0.82^{a}	326.37 ± 0.88^{b}
Pupal fat body	436.23 ± 0.86^{a}	430.75 ± 0.83^{b}
Adult fat body	973.19 ± 0.93^{a}	332.92 ± 0.87^{b}

Means having the different superscript letters differ significantly at P<0.05 in ANOVA (Tukey's test). Data represented as mean± SD, n=6.

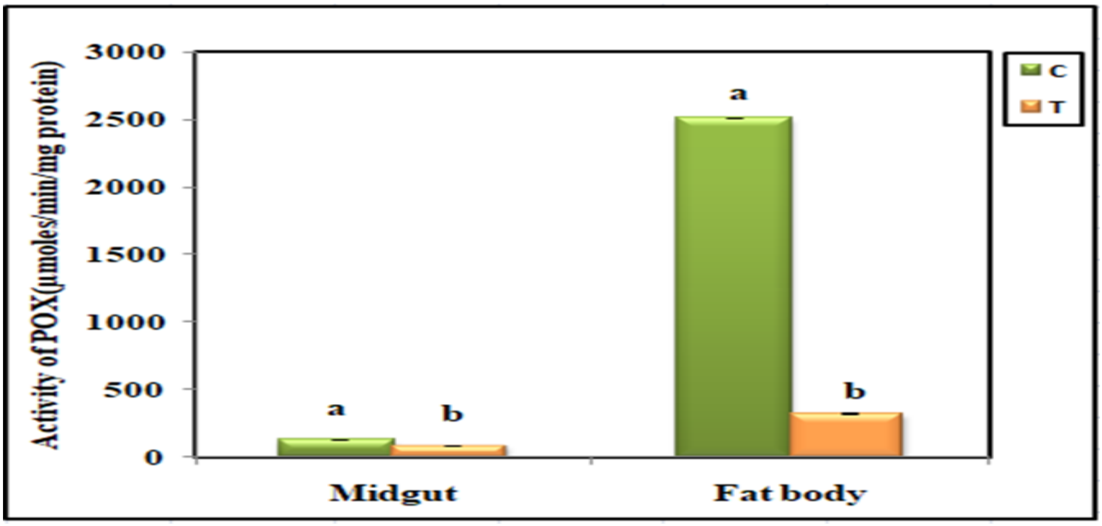
Figure 4.12. POX activity in the larval pupal and adult tissues of control and thermal stress exposed group

(a). The activity of POX in the silk gland of the fifth instar larvae of control and thermal stress exposed group



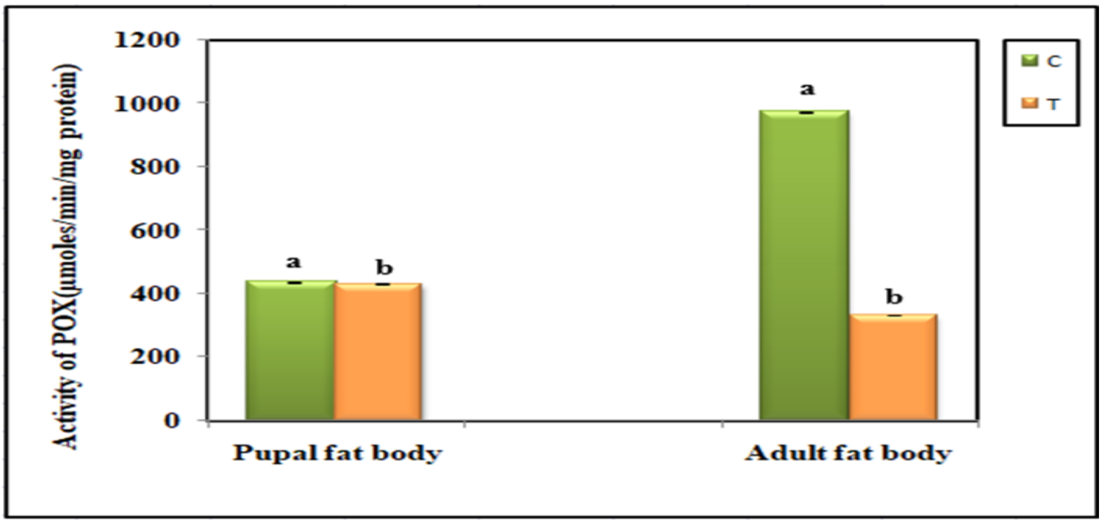
Note : C- Control, T- Thermal stress

(b). Activity of POX in the midgut and fat body of the fifth instar larvae of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

(c). Activity of POX in the fat body of pupa and adult of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

4.4.4. Glutathione S-transferase activity (GST)

GST activity is significantly affected as a result of heat shock exposure in larval, pupal and adult developmental stages of silkworms. Silkworms in the thermal stressed group have significantly increased GST activity in the silk gland (67.57±0.055[a]) and midgut (178.55±0.99[a]) of fifth instar larvae, and fat body of adults. Whereas the fat body of larvae (92.52±0.98[b]) and pupae (38.303±0.635[b]) displayed a significantly diminished GST activity in contrast to the control.

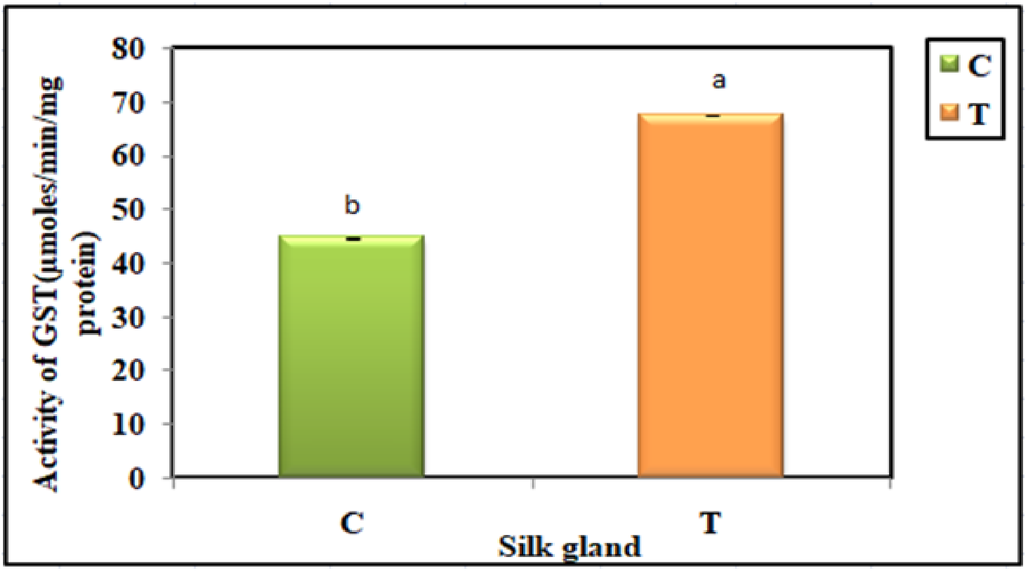
Table 4.10. The activity of GST in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C CONTROL	T THERMAL STRESS
Silk gland	44.68±0.052[b]	67.57±0.055[a]
Midgut	80.45±0.98[b]	178.55±0.99[a]
Fat body	113.61±0.91[a]	92.52±0.98[b]
Pupal fat body	64.7±0.58[a]	38.303±0.635[b]
Adult fat body	214.27±0.53[b]	297.535±0.73[a]

The significance of difference is indicated as by different superscript letters ($P<0.05$, ANOVA, Tukey's test). The values are presented as mean ± SD, n=6.

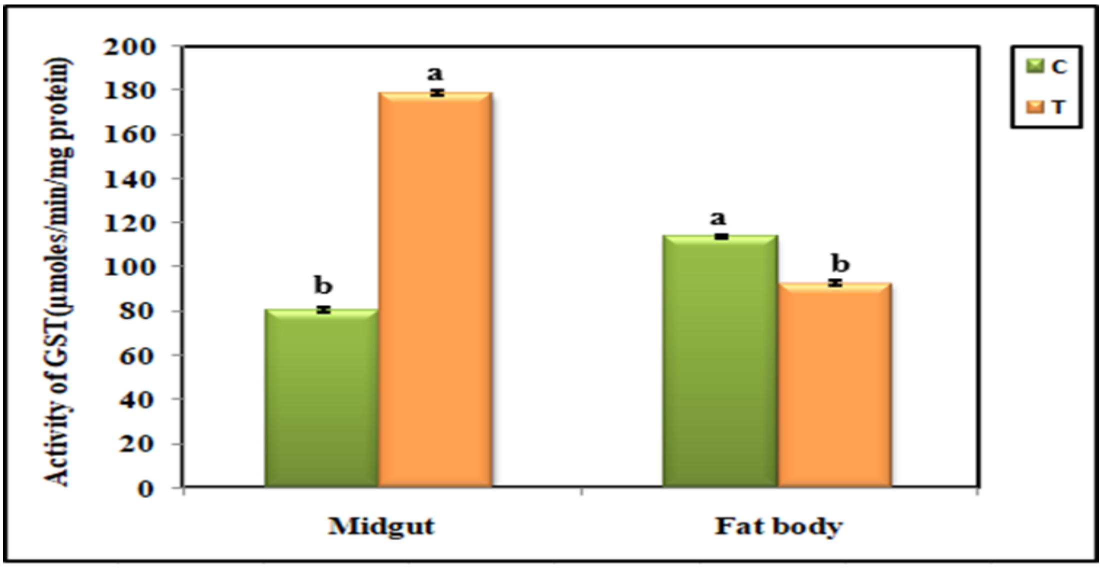
Figure 4.13. GST activity in the larval pupal and adult tissues of control and thermal stress exposed group

(a). The activity of GST in the silk gland of the fifth instar larvae of control and thermal stress exposed group



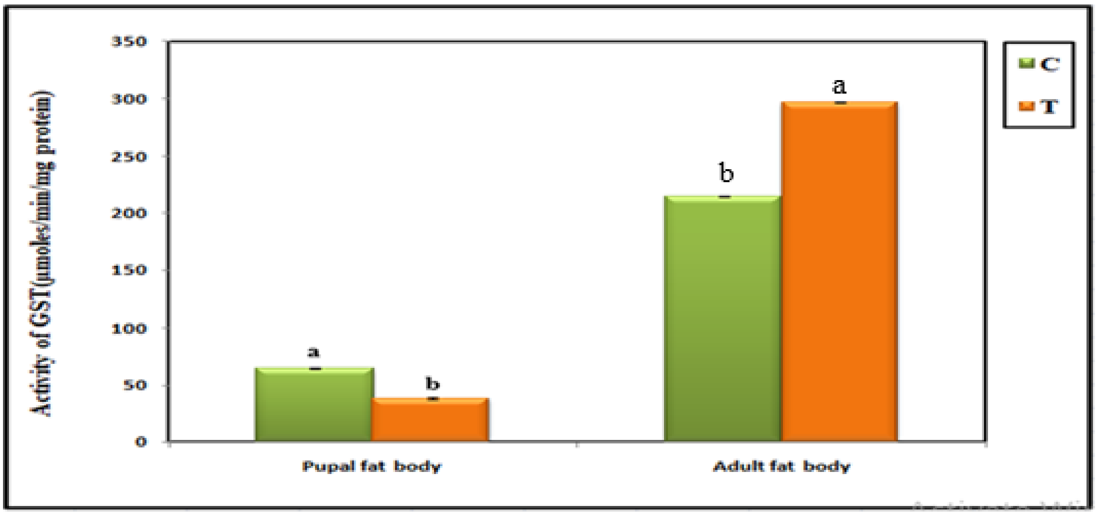
Note : C- Control, T- Thermal stress

(b). Activity of GST in the midgut and fat body of fifth instar larvae of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

(c). Activity of GST in the fat body of pupa and adult of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

4.4.5. Glutathione peroxidase activity (GPx)

Glutathione peroxidase activities in the larval pupal and adult tissues of *B. mori* were persuaded by induced temperature. The larval midgut (228.09±0.9[b]), fat body of pupa (278.65±0.86[b]) and adult (823.31±0.932[b]) showed a reduced GPx activity in the treated group except for silk gland (395.98±0.092[a]) and fat body (501.59±0.91[a]) of larvae in comparison with the control group.

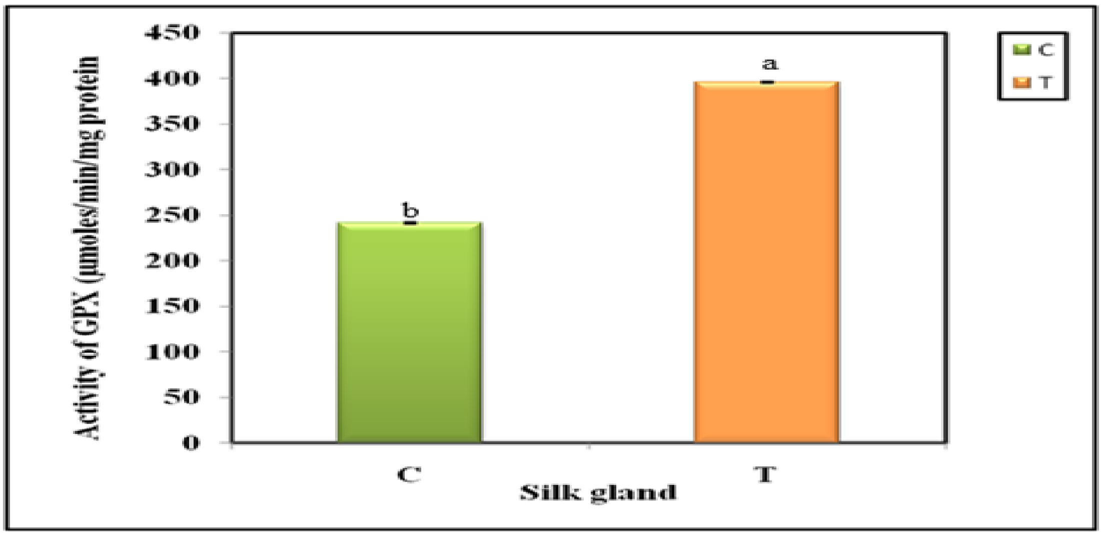
Table 4.11. Activity of GPx in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	241.18±0.103[b]	395.98±0.092[a]
Midgut	232.57±0.54[a]	228.09±0.9[b]
Fat body	285.49±0.82[b]	501.59±0.91[a]
Pupal fat body	294.42±0.94[a]	278.65±0.86[b]
Adult fat body	1211.33±0.98[a]	823.31±0.932[b]

The significance of difference is indicated as by different superscript letters ($P<0.05$, ANOVA, Tukey's test). The values are presented as mean ± SD, n=6.

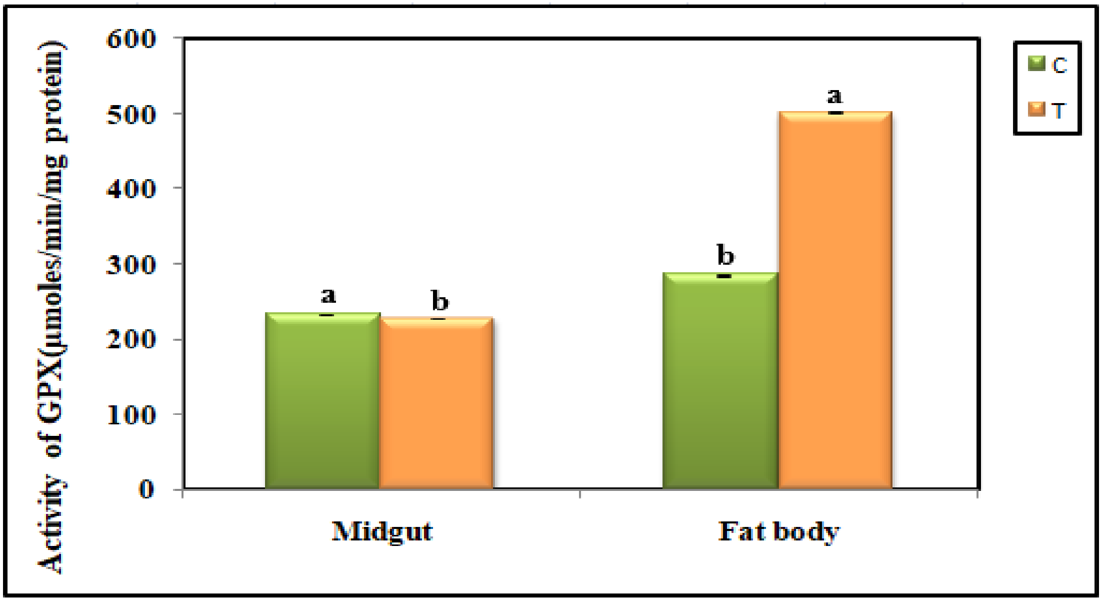
Figure 4.14. GPx activity in the larval pupal and adult tissues of control and thermal stress exposed group

(a). The activity of GPx in the silk gland of the fifth instar larvae of control and thermal stress exposed group



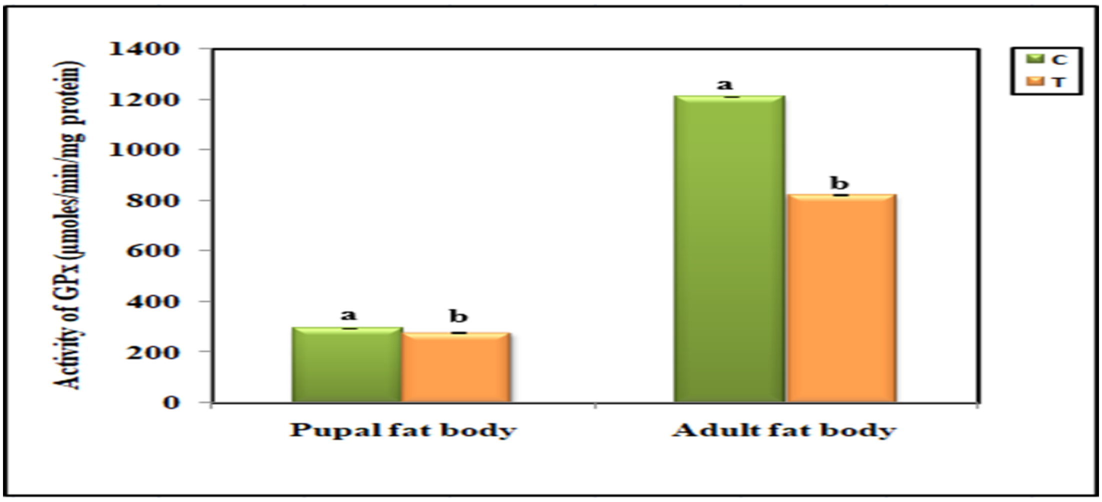
Note : C- Control, T- Thermal stress

(b). Activity of GPx in the midgut and fat body of fifth instar larvae of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

(c). Activity of GPx in the fat body of pupa and adult of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

4.4.6. Glutathione reductase activity (GR)

The activity of the GR in the silk gland (1.312±0.052[a]) of the experimental set showed remarkably elevated activity in contrast to control (0.543±0.068[b]). Similarly the midgut GR activity is increased in the thermal stress exposed group (0.666±0.121[a]) than control (0.463±0.09[b]) group. Thermal stress exposed group fat body (0.88±0. 0.08[a]) of fifth instar larvae implied a similar trend of GR enzyme activity against control (0.55±0.097[b]). Moreover the GR enzyme activity of pupal fat body and adult fat body displayed a significantly higher activity in the thermal stress exposed group than control.

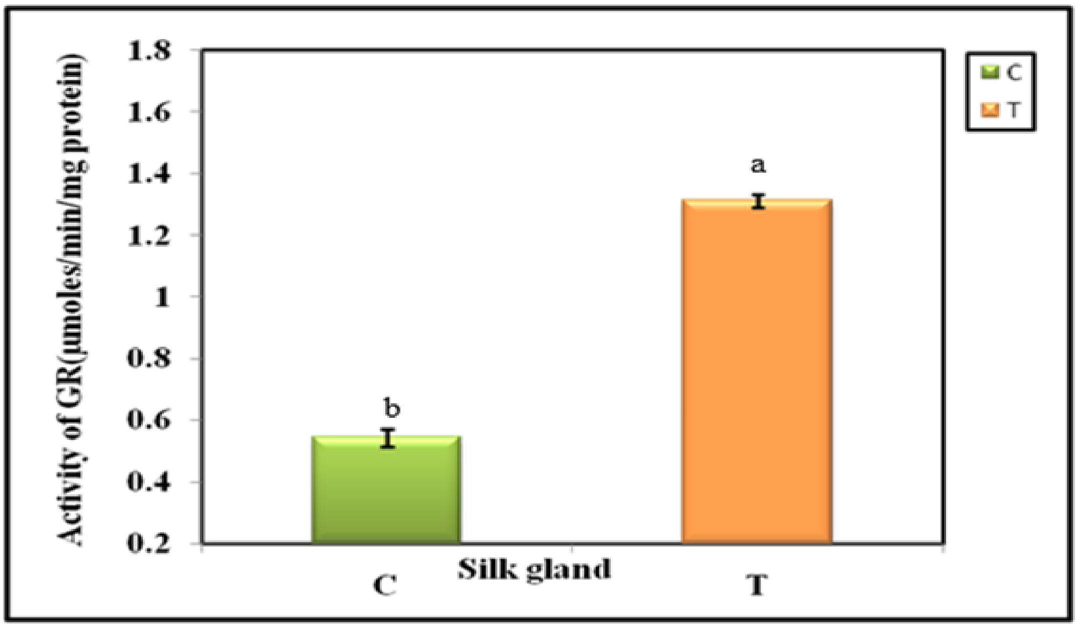
Table 4.12. Activity of GR in different tissues of larva, pupa and adult in control and thermal stress exposed group.

TISSUES	C	T
	CONTROL	THERMAL STRESS
Silk gland	0.543±0.068[b]	1.312±0.052[a]
Midgut	0.463±0.09[b]	0.666±0.121[a]
Fat body	0.55±0.097[b]	0.88±0. 0.08[a]
Pupal fat body	0.27±0.024[b]	0.39±0.06[a]
Adult fat body	3.6±0.17[b]	7.83±0.05[a]

The significance of difference is indicated as by different superscript letters ($P<0.05$, ANOVA, Tukey's test). The values are presented as mean ± SD, n=6.

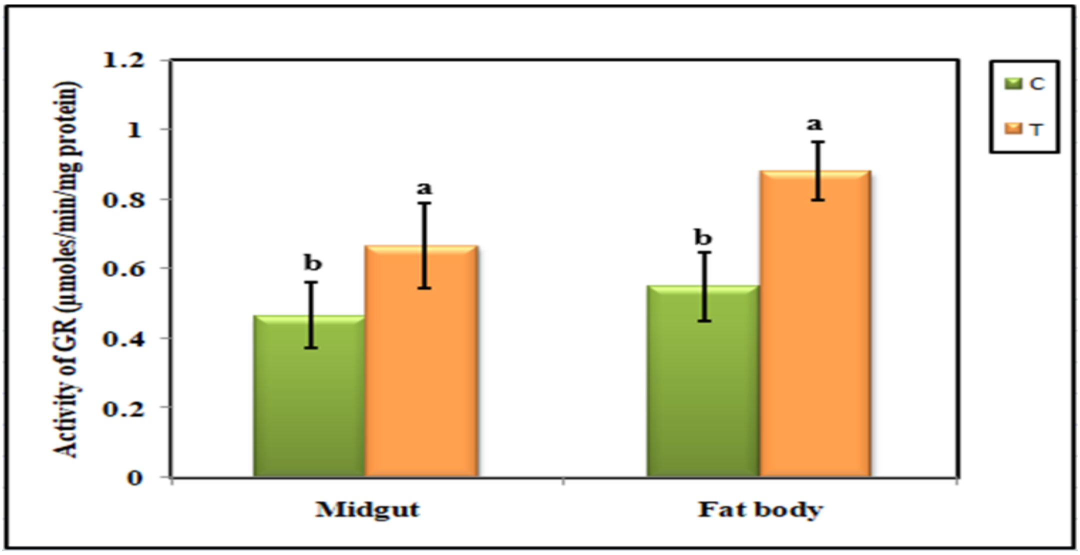
Figure 4.15. GR activity in the larval pupal and adult tissues of control and thermal stress exposed group.

(a). Activity of GR in the silk gland of the fifth instar larvae of control and thermal stress exposed group



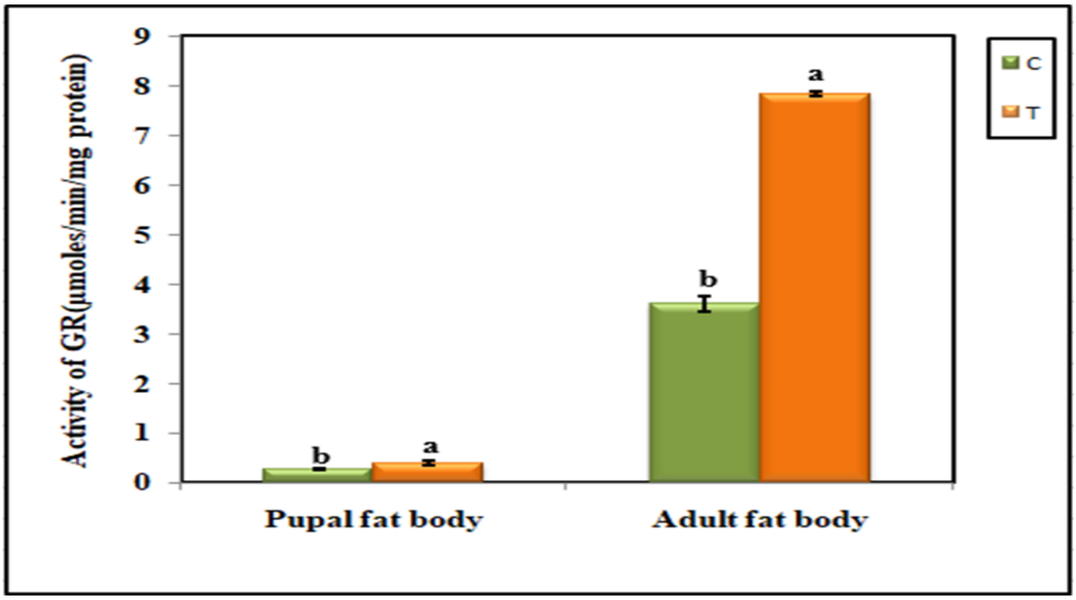
Note : C- Control, T- Thermal stress

(b). Activity of GR in the midgut and fat body of the fifth instar larvae of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

(c). Activity of GR in the fat body of pupa and adult of control and thermal stress exposed group



Note: C- Control, T- Thermal stress

4.5. Molecular analysis

Quantification of relative mRNA expression of BmYki, MBF1, BmApaf-1, BmICE-2, TNFSF5, IL6, Caspase3 and HSP70 genes was done by using qRT PCR of various developmental stages of the silkworm. Fold difference of gene expression analyzed by using 2^-ΔΔCT (Comparative ΔCT method). The significant variation is represented with an asterisk (*) at $P<0.05$ in ANOVA, mean comparisons were done by using Tukey's test.

4.5.1. Tissue and stage specific expression of transcriptional co-activator genes in silkworm

The relative mRNA expression of BmYki and MBF1 genes was analyzed using qRTPCR in the diverse developmental phases of silkworms. Tissue specific expression is executed throughout the silk gland, midgut and fat body of larva, fat body of pupa and fat body of adult in response to thermal stress.

4.5.1.1. Expression analysis of BmYki in diverse developmental phases and tissues

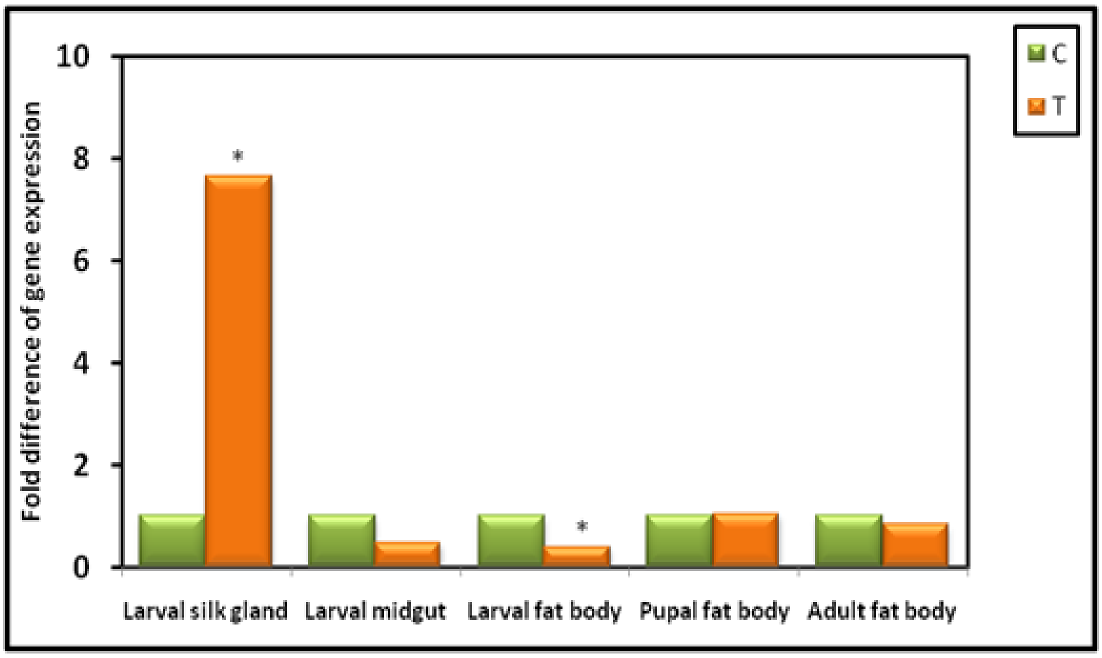
A relative expression analysis of gene BmYki on larval, pupal and adult tissues showed a disparity in the expression in response to thermal stress. The control group exhibited a constant normalized fold expression, mentioned as the value one. When compared to the control, silk gland BmYki expression significantly increased 7.66 fold in the thermal stress exposed group. On the contrary, upon expression analysis, the midgut (0.488), fat body (0.41) and adult fat body (0.83) thermal exposed group showed a decreasing trend of fold expression, in which the fat body of larva exhibited a significant decrease (P<0.05). While the experimental group of the pupal fat body resembles the control (Fig 4.16).

Table 4.13. Expression of BmYki gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Larval silk gland	13.32	12.24667	25.33	27.19	-12.01	-14.95	1	7.66*
Larval midgut	32.92	34.55	28.90	29.07	4.02	5.48	1	0.488
Larval fat body	33.96	35.22	29.57	29.44	4.39	5.78	1	0.41*
Pupal fat body	22.94	24.01667	21.543	22.61	1.397	1.4	1	1.05
Adult fat body	22.90	24.39	22.18	23.38	0.72	1.01	1	0.83

Note: C- Control, T-Thermal stress. Data are shown as the mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.16. Expression of the BmYki gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.1.2. Expression analysis of MBF1 in diverse developmental phases and tissues

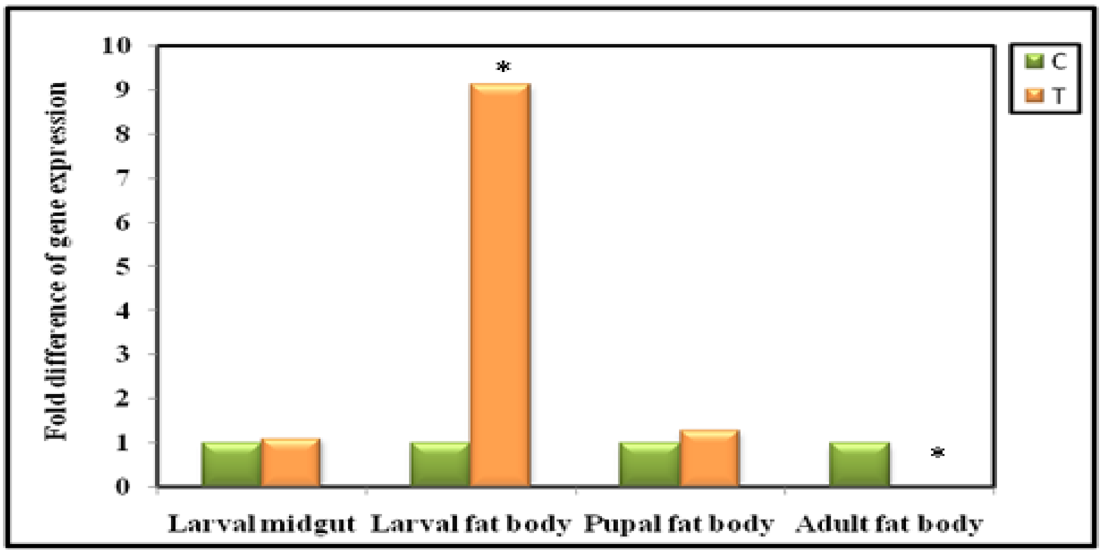
The results of MBF1 gene expression in the silkworm tissues such as the midgut, and fat body of larva, pupal and adult fat body, could be detected in all tissues. The control group exhibited a constant normalized fold expression, mentioned as the value one. In group II fifth instar larval tissue fat body (9.14) displayed a drastic and significant ($P<0.05$) upregulation of the MBF1 gene, while other tissues such as the fifth instar midgut (1.13) and pupal fat body (1.3) also showed an upregulation trend but it is not showing any significant difference. On the contrary, adult fat body (0.021), in group II exhibited a significant decline, compared to the control group.

Table 4.14. Expression of MBF1 gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Larval midgut	8.70	9	28.9	29.07	-20.2	-20.07	1	1.13
Larval fat body	9.82	6.5	29.57	29.44	-19.75	-22.94	1	9.14*
Pupal fat body	20.90	21.69	21.54	22.61	-0.64	-0.92	1	1.3
Adult fat body	17.64	24.66	22.18	23.38	-4.54	1.28	1	0.021*

Note: C- Control, T-Thermal stress. Values are shown as the mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.17. Expression of MBF1 gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.2. Tissue and stage specific expression of pro-apoptotic genes in silkworm

BmApaf-1 and BmICE-2 are pro-apoptotic genes, and the expression pattern of these genes is investigated in the different developmental stages of silkworms.

4.5.2.1. Expression analysis of BmApaf-1 in diverse developmental phases and tissues

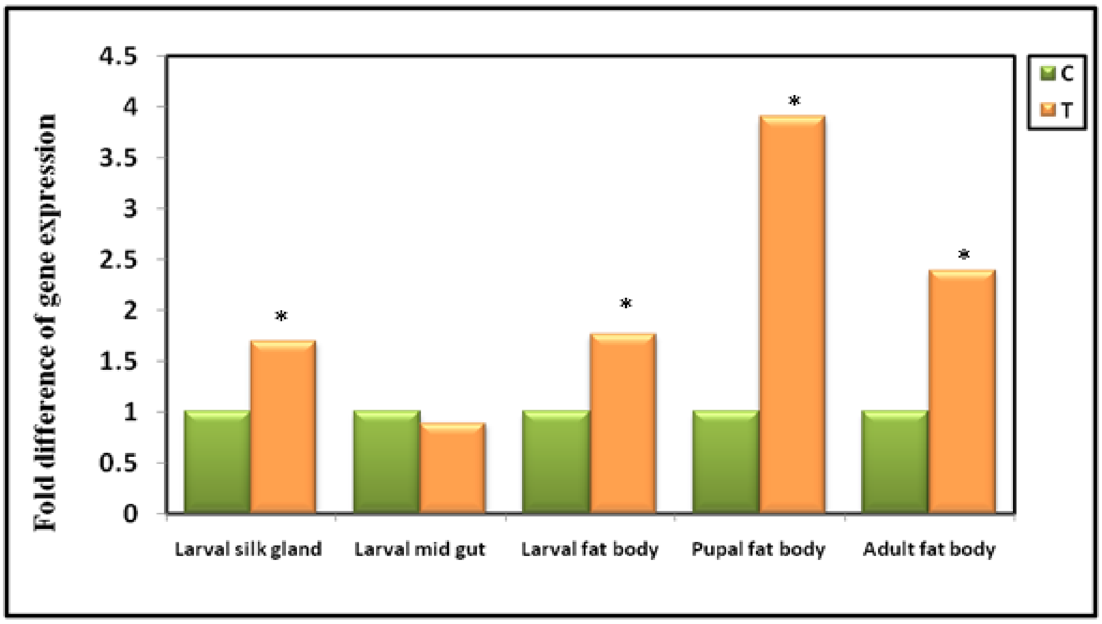
The examination of the Apaf-1 gene expression in diverse developmental stages of silkworms revealed the presence of this gene in all the tissues studied and developmental stages. The larval silk gland (1.701) and fat body (1.763), pupal fat body (3.894) and adult fat body (2.391) exhibited significantly ($P<0.05$) upregulated fold expression in the test group with respect to untreated group. Whereas the midgut of the fifth instar larva showed a down regulated Apaf-1 expression subsequent to heat exposure. Among the all tissues, the pupal fat body exhibited a greatest fold difference of gene expression in thermal stress exposed group in comparison to the control.

Table 4.15. Expression of BmApaf-1 gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Larval silk gland	30.67	29.20	30.35	29.64	0.32	-0.44	1	1.701*
Larval midgut	29.44	29.92	28.9	29.07	0.54	0.85	1	0.8943
Larval fat body	30.88	30.12	29.68	29.61	1.2	0.51	1	1.763*
Pupal fat body	22.75	21.98	21.54	22.61	1.21	-0.63	1	3.894*
Adult fat body	23.87	23.82	22.183	23.383	1.687	0.437	1	2.391*

Note: C- Control, T-Thermal stress. Data represented as mean ± SD (n=3).The significant variation is represented with an asterisk (*) at $P<0.05$ in ANOVA (Tukey's test).

Figure 4.18. Expression of BmApaf-1gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.2.2. Expression analysis of BmICE-2 in diverse developmental phases and tissues

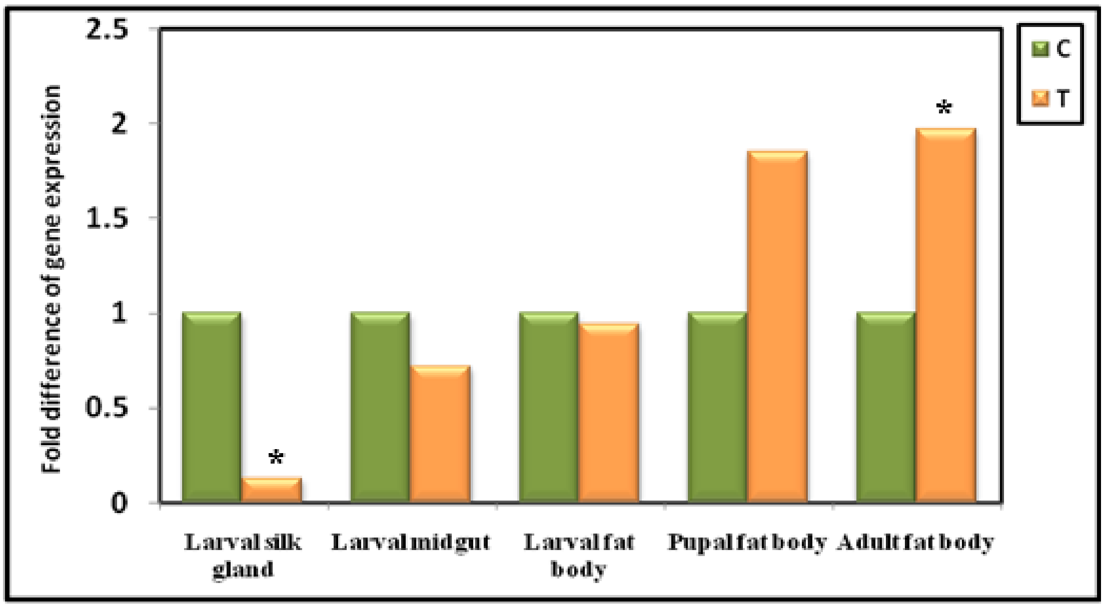
In the silk gland, midgut and fat body of larva, the BmICE-2 showed a down regulated expression profile in the experimental group, but the developmental stage pupa and adult showed an upregulated trend of fold expression of BmICE-2 against the control. The adult fat body exhibited a significant ($P<0.05$) increase in gene expression in group II (T), and the silk gland showed a significant decrease of BmICE-2 in the thermal stress exposed group.

Table 4.16. Expression of BmICE-2 gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Larval silk gland	25.84	32.38	26.16	27.19	-0.31	5.18	1	0.13306*
Larval midgut	32.05	32.86	28.90	29.07	3.15	3.79	1	0.72
Larval fat body	28.91	28.96	29.57	29.44	-0.66	-0.48	1	0.941
Pupal fat body	23.15	23.49	21.54	22.61	1.61	0.87	1	1.85
Adult fat body	24.82	24.965	22.18	23.295	2.64	1.67	1	1.965*

Note: C- Control, T-Thermal stress. Data are represented as mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.19. Expression of BmICE-2 gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.3. Tissue and stage specific expression of inflammatory response genes in silkworm

The inflammatory response genes IL6 and TNFSF5 expression were examined in the larval pupal and adult stages of silkworms. In this study, IL6 and TNFSF5 expression were investigated in the silkworm tissue and haemolymph samples such as larval haemolymph and fat body, pupal haemolymph, pupal fat body and adult fat body.

4.5.3.1. Expression analysis of IL6 in diverse developmental phases and tissues

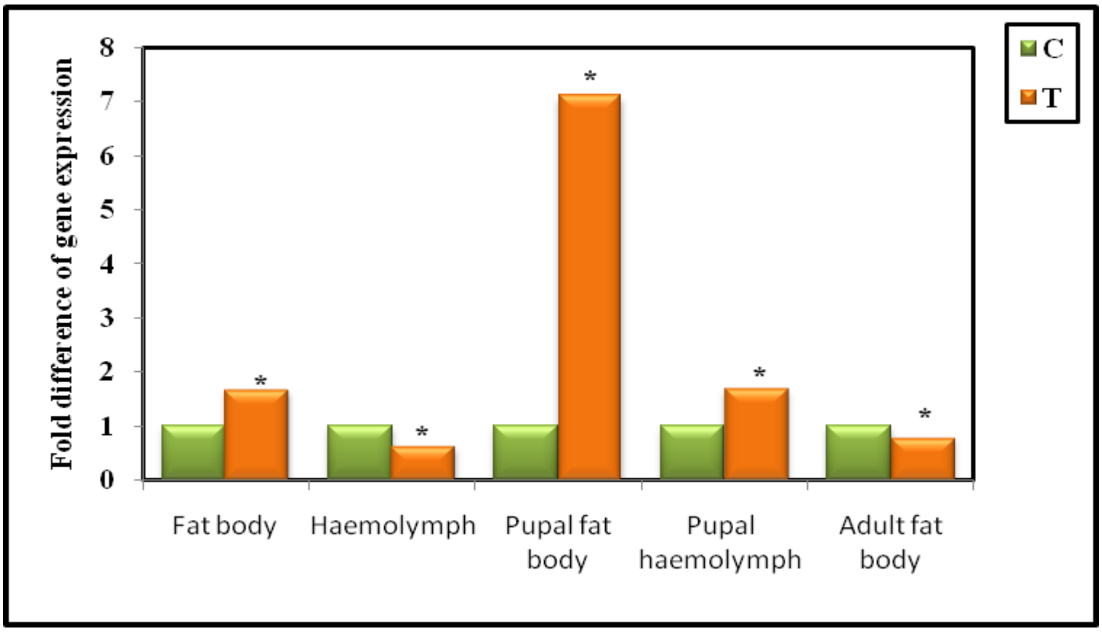
All the investigated tissues and haemolymph samples showed the presence of the IL6 gene. The IL6 expression levels in the thermal stress exposed group of the fat body showed 1.68 fold significant (P<0.05) upregulation of gene expression, whereas haemolymph of larva displayed a 0.641 fold of significantly decreased IL6 expression in the test group compared with control. The pupal fat body (7.103) and pupal haemolymph (1.7) also showed significantly increased fold gene expression in the thermal stress exposed group. Adult fat body displayed a significantly decreasing trend of gene expression when exposed to temperature stress.

Table 4.17. Expression of IL6 gene in diverse developmental phases and tissues of silkworm under thermal stress

SAMPLES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Fat body	28.83	27.85	29.87	29.63	-1.04	-1.78	1	1.68*
Haemolymph	28.23	28.13	29.27	28.44	-1.04	-0.31	1	0.641*
Pupal fat body	20.94	19.34	21.51	22.73	-0.57	-3.39	1	7.103*
Pupal haemolymph	28.60	28.38	28.37	28.88	0.23	-0.50	1	1.7*
Adult fat body	20.71	22.24	22.18	23.38	-1.47	-1.14	1	0.807*

Note: C- Control, T-Thermal stress. Data represented as mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.20. Expression of IL6 gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.3.2. Expression analysis of TNFSF5 in diverse developmental phases and tissues

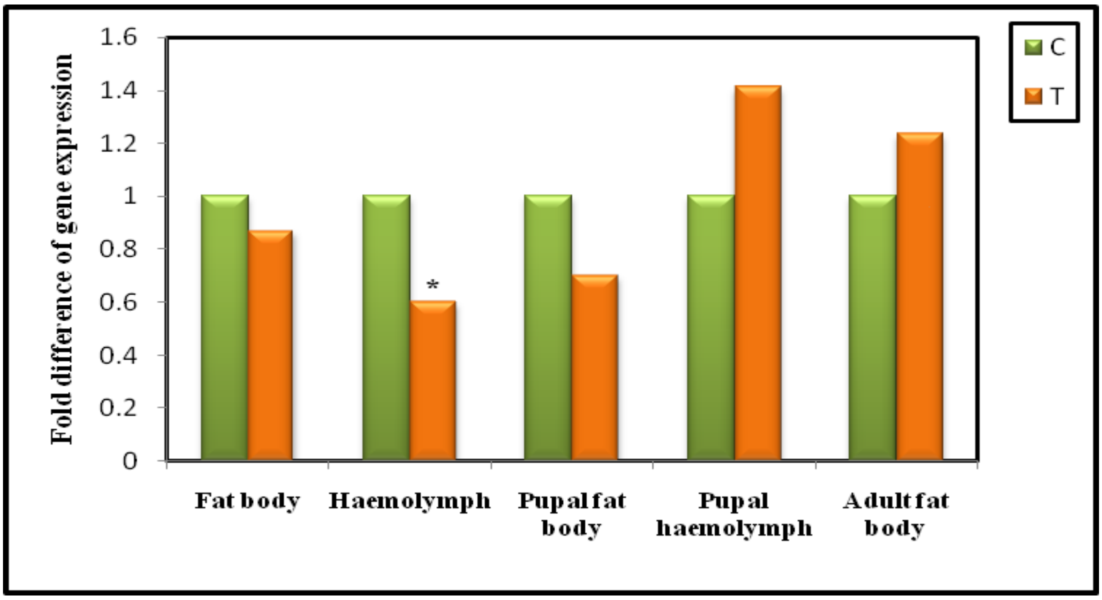
The larval haemolymph and fat body exhibited a similar trend of gene expression. However a key difference was observed for TNFSF5, its expression in the haemolyph of the temperature exposed group showed a significant decline in fold expression, while its expression in the fat body of the exposed group was not affected significantly. In the pupal and adult stages, pupal haemolymph and adult fat body showed the same pattern of expression among the heat shock exposed group but were not significant. Pupal fat body expression patterns share similarities with larval haemolymph and fat tissue.

Table 4.18. Expression of TNFSF5 gene in diverse developmental phases and tissues of silkworm under thermal stress

SAMPLES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Fat body	32.41	32.50	29.57	29.44	2.84	3.06	1	0.87
Haemolymph	32.55	32.53	29.27	28.45	3.28	4.08	1	0.6011*
Pupal fat body	26.64	28.27	21.54	22.61	5.10	5.66	1	0.702
Pupal haemolymph	32.43	32.26	28.53	28.69	3.9	3.57	1	1.411
Adult fat body	28.25	29.15	22.18	23.38	6.07	5.77	1	1.2343

Note: C- Control, T-Thermal stress. Data represented as mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.21. Expression of TNFSF5 gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.4. Tissue and stage specific expression of caspase3 gene in silkworm

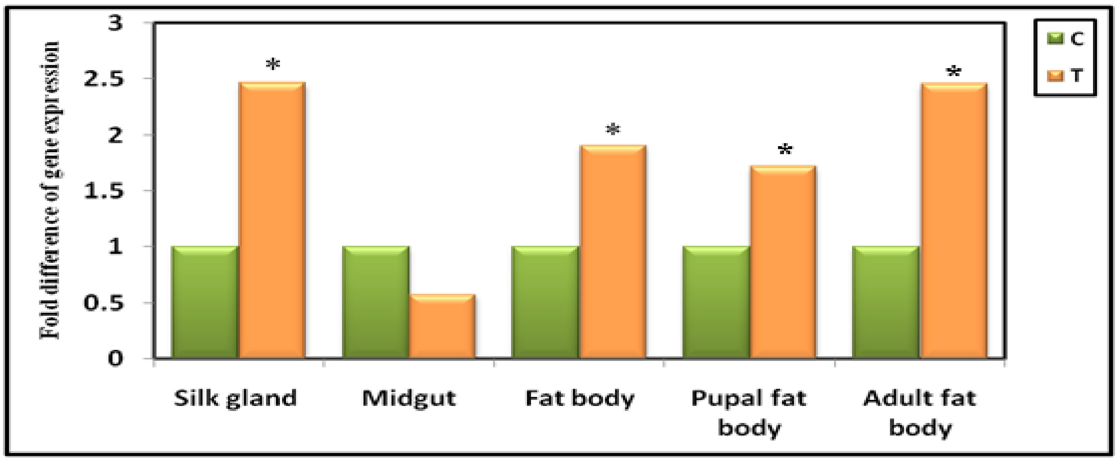
The presence of the caspase3 gene was detected in all the investigated tissue samples. The silk gland (2.476 fold) and adult fat body (2.458 fold) showed the highest gene expression compared to other tissues when exposed to heat shock. The fat body of pupa and larvae displayed more or less similar significant (P<0.05) gene expression in the thermal stress exposed group. On the contrary, the midgut gene expression decreased in the test group but it is not statistically significant.

Table 4.19. Expression of caspase3 gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Silk gland	26.73	26.35	26.16	27.19	0.57	-0.84	1	2.476*
Midgut	29.87	30.21	29.56	29.07	0.31	1.14	1	0.5813
Fat body	31.47	30.51	29.57	29.44	1.9	1.07	1	1.9118*
Pupal fat body	30.53	30.85	21.54	22.61	8.99	8.24	1	1.721*
Adult fat body	25.52	25.45	22.183	23.38	3.337	2.07	1	2.458*

Note: C- Control, T-Thermal stress. Data represented as mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.22. Expression of caspase3 gene in response to thermal stress



Note: C- Control, T- Thermal stress

4.5.5. Tissue and stage specific expression of Hsp70 gene in silkworm

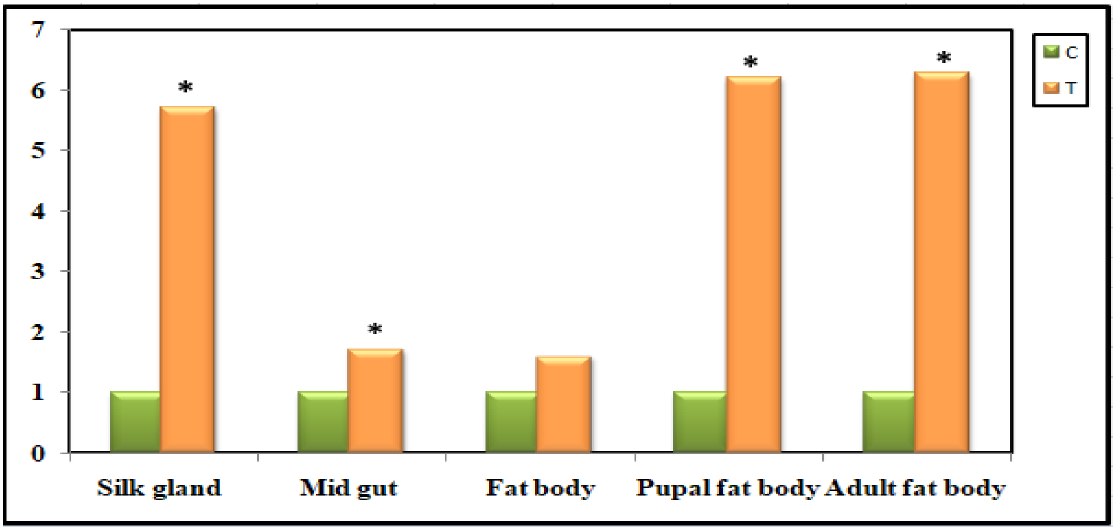
Gene expression analysis of Hsp 70 displayed a significantly (P<0.05) upregulated fold gene expression in the silk gland, and midgut of larva, fat body of pupa and adult when compared with the control. The fat body of the larva showed an upregulated expression but it is not significant.

Table 4.20. Expression of Hsp70 gene in diverse developmental phases and tissues of silkworm under thermal stress

TISSUES	Mean Ct		BmActin		Normalized expression value		Fold difference	
	C	T	C	T	C	T	C	T
Silk gland	24.26	22.43	23.77	24.46	0.49	-2.03	1	5.72*
Midgut	28.33	28.49	28.39	29.32	-0.06	-0.83	1	1.71*
Fat body	28.49	27.73	29.57	29.44	-1.08	-1.71	1	1.594
Pupal fat body	20.63	19.52	21.54	22.71	-0.91	-3.19	1	6.22*
Adult fat body	22.17	20.57	22.18	23.33	-0.01	-2.76	1	6.29*

Note: C- Control, T-Thermal stress. Data represented as mean ± SD (n=3). The significant variation is represented with an asterisk (*) at P<0.05 in ANOVA (Tukey's test).

Figure 4.23. Expression of the Hsp70 gene in response to thermal stress



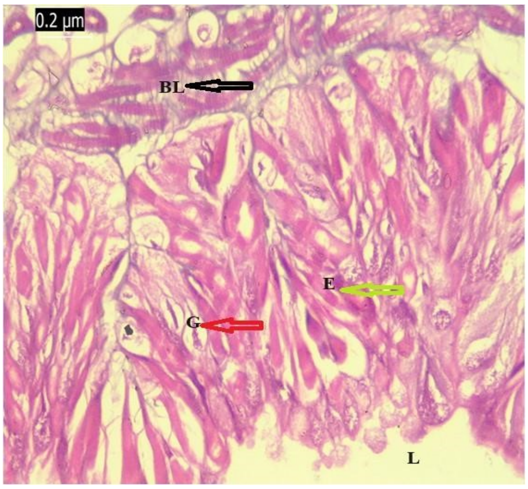
Note: C- Control, T- Thermal stress

4.6. Histopathology of larval tissue

The histopathological observation showed that the epithelial cells and goblet cells have a definite and ordered arrangement in the larval midgut of the control group. The basal lamina is the basic backbone structure from which the epithelial cells and goblet cells hang to the lumen of the midgut and form a completely intact structure. Conversely, the histopathological examination of larval midgut under heat exposed conditions showed that heat stress affects the morphology and structural organization of midgut cells. The goblet cells increased in size and blended with epithelial cells which formed an erratic arrangement of midgut cells.

Figure 4.24. Histopathology of larval midgut of control and treated group

a.



b.

Figure 4.24.a. Represents the control midgut and figure 4.24.b. Stands for the experimental group of midgut under 40x microscopic image with a scale bar showing 0.2µm. BL represents the basal lamina; G stands for goblet cells; E represents epithelial cells and L represents the lumen of the midgut.

4.7. Quantity and quality assessment of cocoon

Table 4.21. Quality of cocoons and cocoon filament of control and experimental group

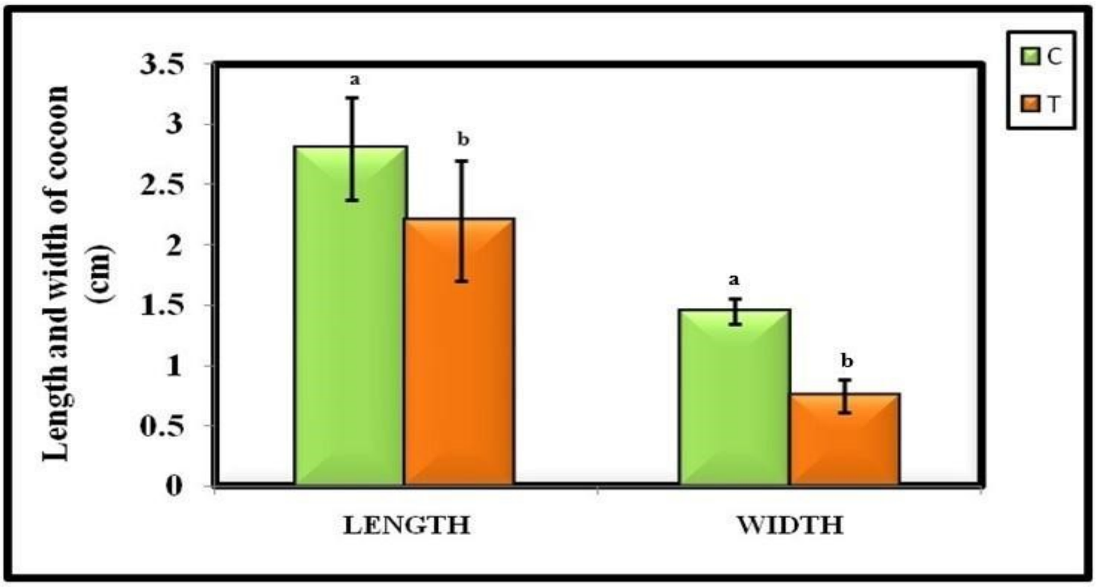
COCOON CHARACTERS	C	T
	CONTROL	THERMAL STRESS
Mean length of cocoon	2.8±0.424[a]	1.45±0.494[b]
Mean width of cocoon	2.2±0.105[a]	0.75±0.138[b]
Average single cocoon weight	1.720±0.026[a]	1.165±0.026[b]
Cocoon shell weight	0.378±0.0072[a]	0.177±0.0072[b]
Cocoon shell ratio	21.97±0.34[a]	15.19±0.593[b]
Filament length	762.7±2.805[a]	585.833±2.7[b]
Filament weight	0.3±0.0232[a]	0.11±0.027[b]
Denier of the cocoon	3.483±0.28[a]	1.633±0.3729[b]
Reelability percentage	88.33±0.69[a]	57.62±1.27[b]

Means having the different superscript letters differ significantly at $P<0.05$ in ANOVA (Tukey's test). Data represented as mean± SD, n=6.

4.7.1. Length and width of the cocoon

The impact of thermal stress on the average length and width of the cocoon was studied and analyzed; the mean length of the cocoon had been considerably reduced in the test group contrasted with control group, which indicates the effect of thermal stress on the cocoon characters and silk production. Significant difference calculated using ANOVA followed by Tukey's test.

Figure: 4.25. The average length and width of the cocoon in the control and experimental group

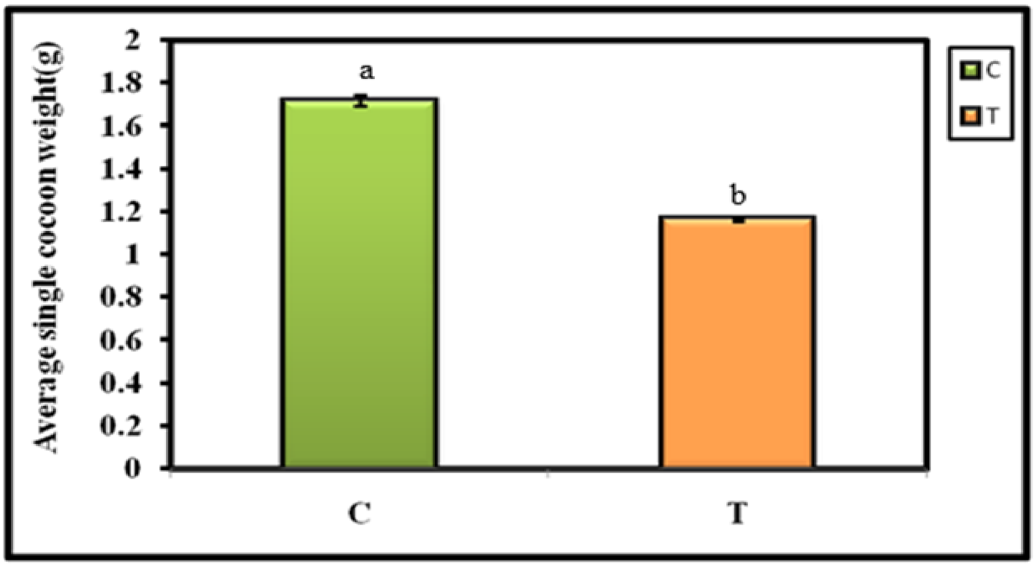


Note: C- Control, T- Thermal stress

4.7.2. Average single Cocoon weight (g)

The average single cocoon weight (1.720±0.026) had been strikingly reduced in the test group contrary to control (1.165±0.009). This result revealed that thermal stress directly affects the production of good quality cocoons.

Figure: 4.26. Average single cocoon weight of control and experimental group

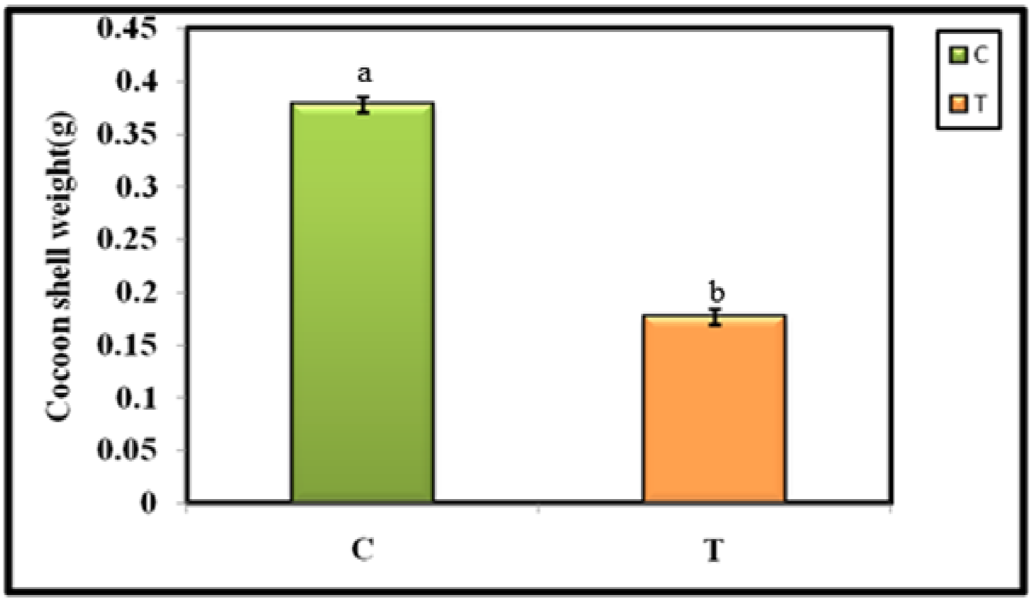


C- Control, T- Thermals tress

4.7.3. Cocoon shell weight (g)

The shell weight of the control and treated groups were calculated and a remarkable abatement in the shell weight of the cocoon in the heat exposed group contrasted with control. The cocoon shell weight was 0.378±0.0072 in group control which is a baseline measurement under standard conditions, the test group was 0.177±0.0072.

Figure: 4.27. The shell weight of the cocoon of control and experimental group

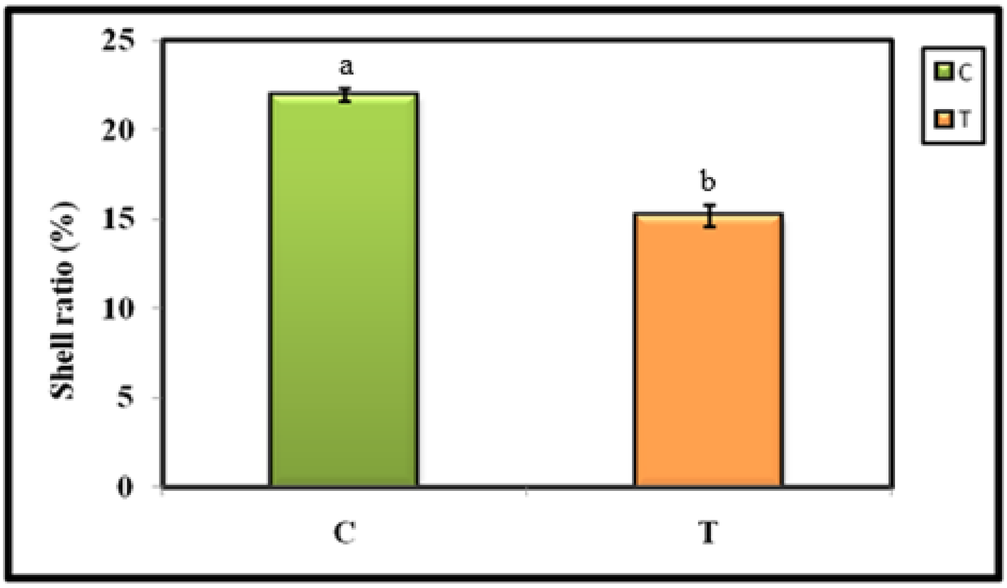


Note: C- Control, T- Thermal stress

4.7.4. Cocoon shell percentage (%)

The cocoon shell percentage of control was 21.97±0.34 which is taken as a guideline value at normal temperature conditions. In the test group cocoon shell percentage was reduced to 15.19±0.593, which led to focus attention on the effect of thermal stress on the cocoon shell percentage.

Figure: 4.28. Cocoon shell percentage of control and experimental group

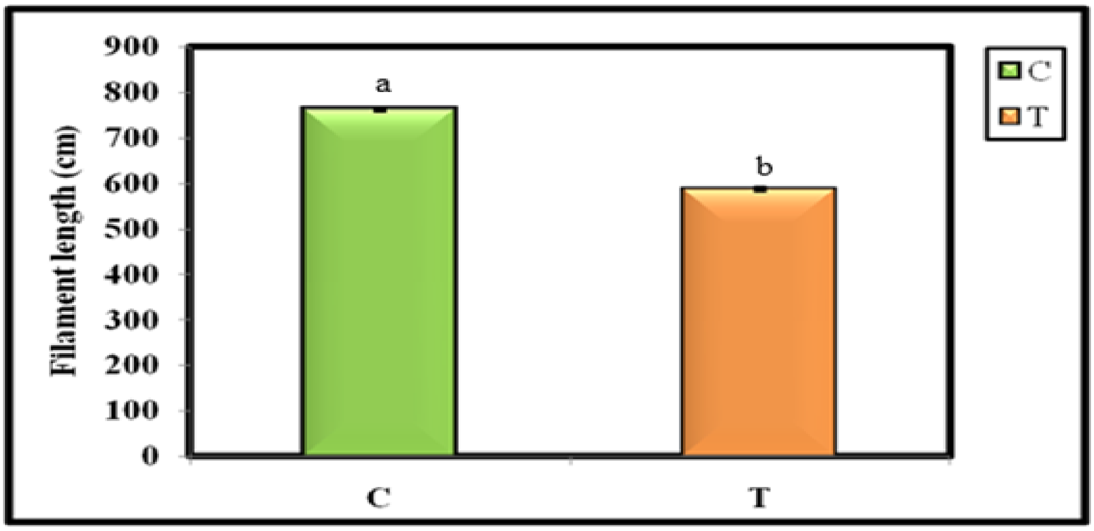


Note: C- Control, T- Thermal stress

4.7.5. Filament length(cm)

Filament length of the control group was measured against the thermal stress exposed group and those two values showed a wide disparity between each group. The average filament length of the control group was 762.7±2.805; the test group was reduced to 585.833±2.7, this notable decrease in filament length indicates the impact of thermal stress on the cocoon production.

Figure: 4.29. Filament length of the cocoon of control and experimental group

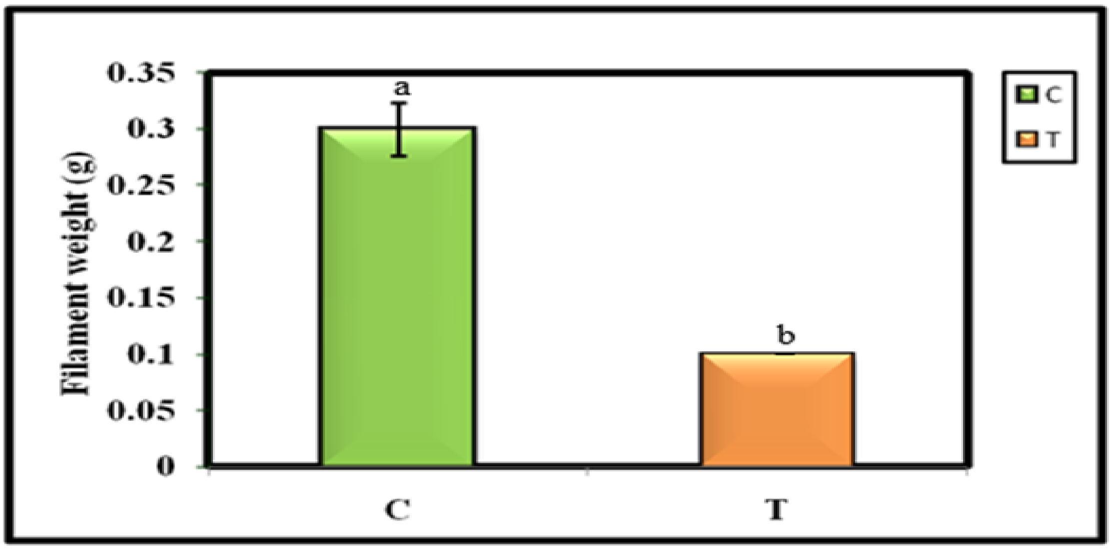


Note: C- Control, T- Thermal stress

4.7.6. Filament weight

Filament weight is one of the quantitative factors which determine the quality of cocoons as well as the quality of silk filament. In the heat shock group filament weight was reduced to 0.11±0.027, compared to the control group (0.3±0.0232) reflecting the effect of heat shock.

Figure: 4.30. Filament weight of the cocoon of control and experimental group

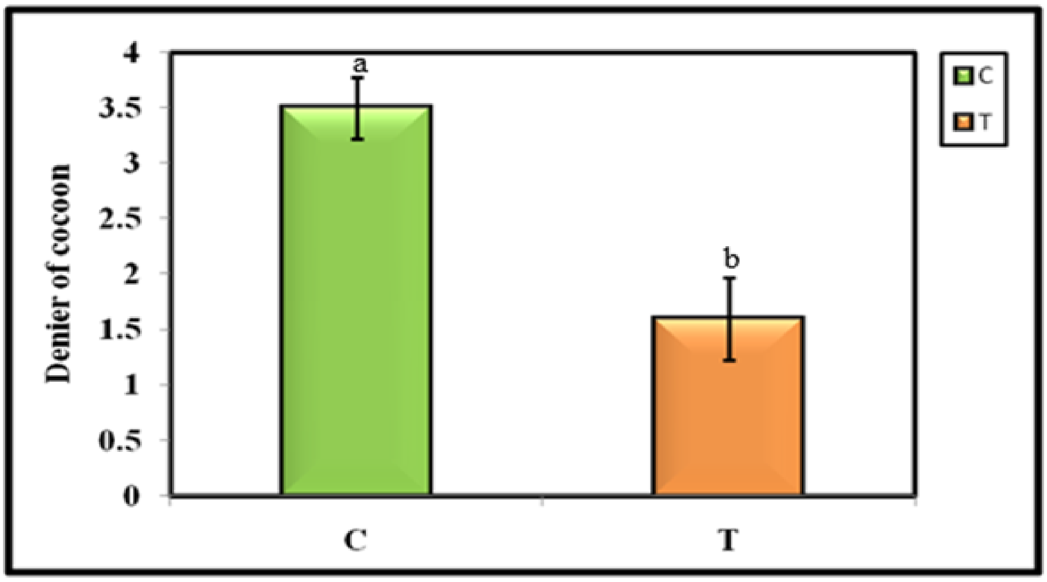


Note: C- Control, T- Thermal stress

4.7.7. Denier of the Cocoon

The size of the cocoon declined in the test group in response to temperature exposure, which confers an idea about the sequel of temperature exposure on the production of the cocoon. The control group cocoon size was 3.483±0.28 at ambient temperature while the test group cocoon size was diminished to 1.633±0.3729.

Figure: 4.31. Denier of the cocoon of control and experimental group

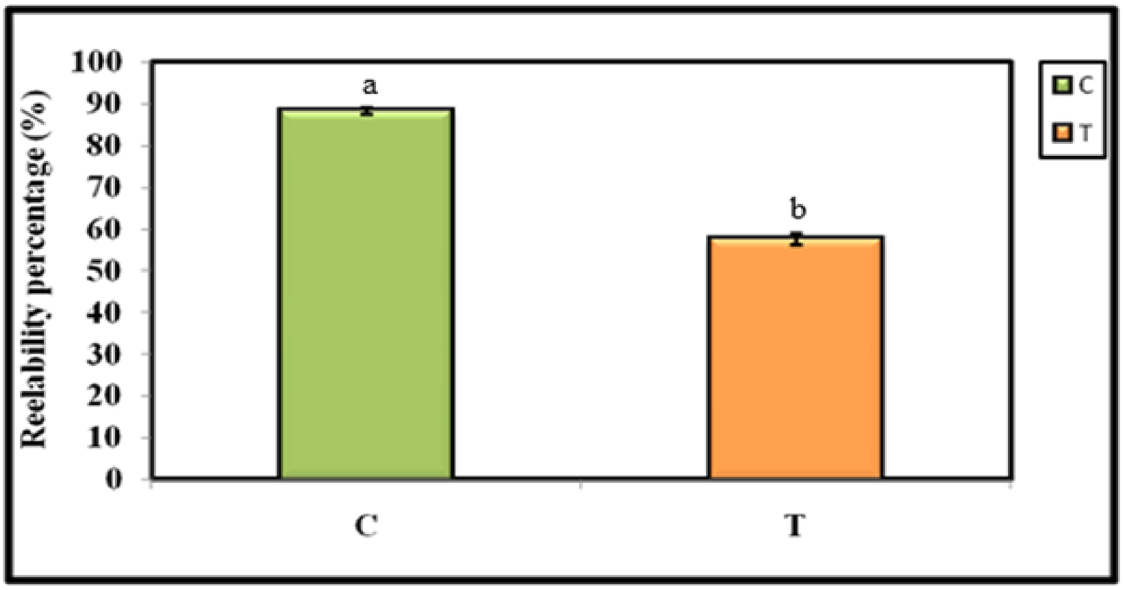


Note: C- Control, T- Thermal stress

4.7.8. Reelability Percentage

Silk reeling is the process of production of a single thread of silk from a large number of cocoon baves reeled together. The control group (88.33±0.69) established an uppermost percentage of reelability than the test group (57.62±1.27).

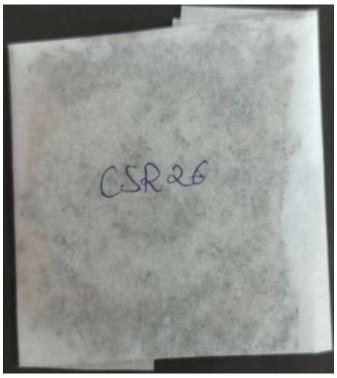
Figure: 4.32. Reelability percentage of the cocoon of control and experimental group



Note: C- Control, T- Thermal stress

PLATE 1

Developmental stages of silkworm *Bombyx mori*



Egg card

Egg hatching

First instar larvae

Second instar larvae

Third instar larvae

Fourth instar larvae

Fifth instar larvae

Cocoon

Pupae

Male adult

Female adult

Mating

PLATE 2

Collapsible plastic mountages for cocoon formation

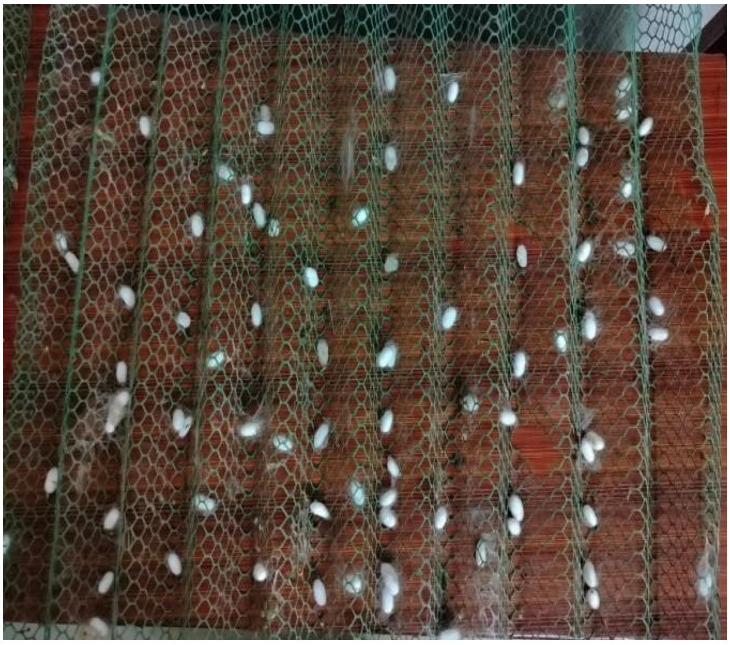
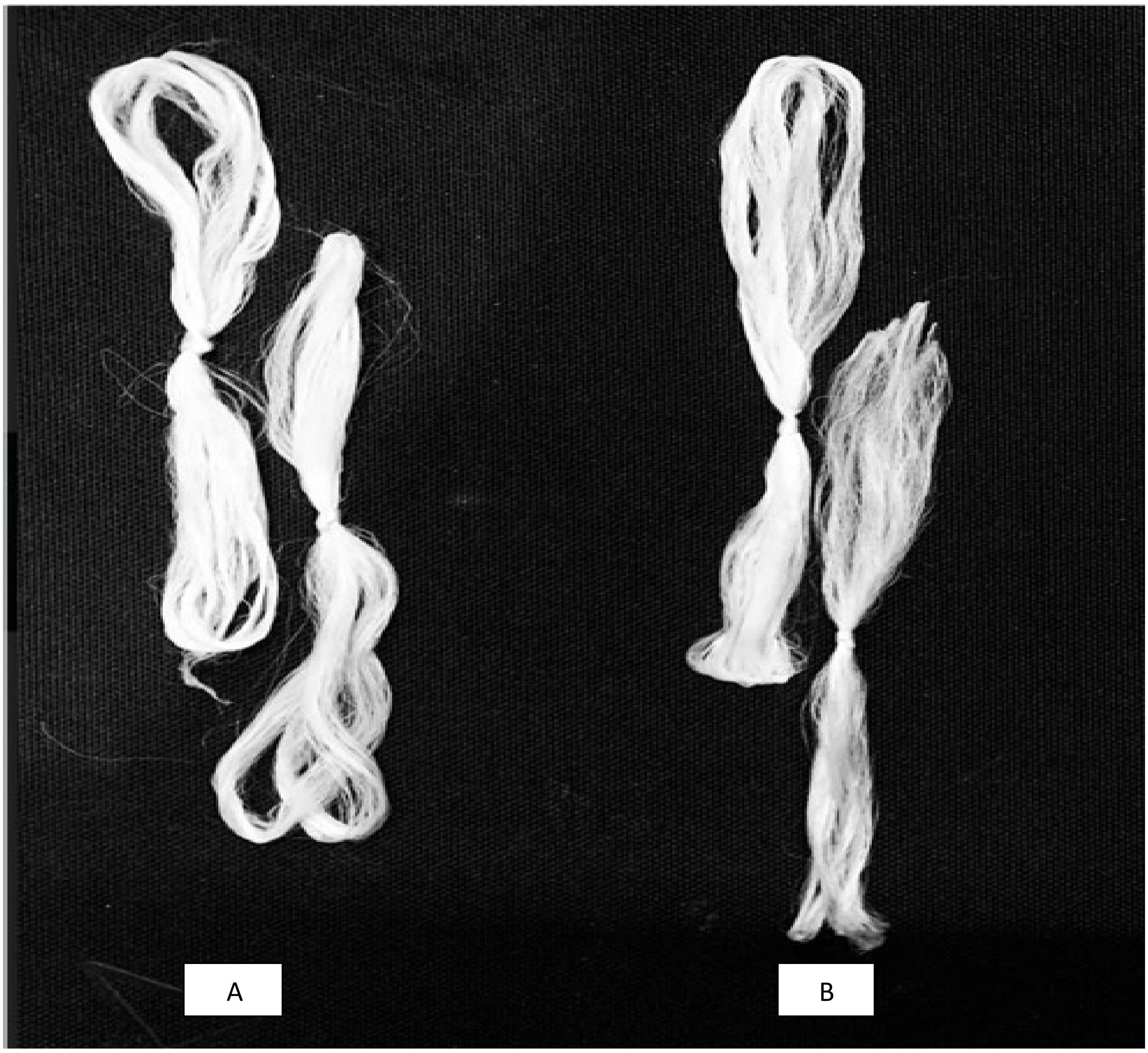


PLATE 3

Silk filaments reeled from cocoons of control and experimental group



A. Control

B. Experimental group

PLATE 4

(a) The formation of brown colour on the body of fifth instar larvae and the inability to spin a cocoon under thermal stress

(b) Dead larvae on exposure to thermal stress

(c) Incomplete pupal development due to thermal stress

(d) Dead pupae on exposure to thermal stress

(e) Thin shelled cocoon formation on exposure to thermal stress

DISCUSSION

5. DISCUSSION

The rise in environmental temperature due to global warming poses a serious menace to insect populations. This is because elevated temperature can alter and impair their normal physiological activities. The environmental conditions pose a great involvement in the growth and development of silkworms. The change in the ambient temperature results in negative effects on the cocoon production and biology of silkworm (Rahmathulla, 2012). High temperature is a major factor that leads to the diminishing of sericulture production.

Insects subjected to protracted high temperatures experience irreversible damage (Li *et al*., 2023). The resulting temperature stress triggers the overproduction of reactive oxygen species, which causes oxidative stress. This stress, in turn, imparts damage by oxidizes lipids, degrades proteins, inactivates enzymes, and eventually leads to cellular demise (Bryan *et al*., 1993). Mitochondrion is the major source of ROS production. ROS triggers apoptosis by the activation of mitochondrial dependent and independent apoptotic pathways (Anderson *et al*., 1999). Information regarding ROS induced apoptosis or programmed cell death is ambiguous in insects. However, this study aims to determine how thermal stress influences the expression of genes associated with apoptosis, transcriptional co-activators, and inflammatory responses. The study also examined the repercussion of heat shock on the histopathological variations and biochemical activities of silkworm larvae, pupae and adults and also the quality cocoon production.

5.1. Morphometric parameters of silkworm

Silkworm *Bombyx mori* is a thermal sensitive organism, known for producing luxuriant silk thread. Consequently, its growth and development are greatly influenced by the environmental temperature. Alterations in the environmental temperature directly affect the silkworm growth and development (Tanjung *et al*., 2017). The larval food consumption, utilization and development depend on different biotic and abiotic factors (Rahmathulla and Devi, 2001).

As the fifth instar is the silkworm's active feeding phase, exposure to high temperatures during this period eventually compromises the consumption of mulberry leaves. The conversion rate of leaf to silk is diminished with increasing temperature from 20 to 30ºC (Takeuchi *et al*., (1964); Muniraju *et al*., (1999). The reduction in the feeding ultimately affects the larval health, which results in an unhealthy pupa and finally it will lead to the reduction in the quality and quantity of cocoon production.

In the present study the larva, pupa and adult exposed to high temperature results in a significant change in the morphological parameters. The experimental group of larvae, pupa and adult revealed a significant reduction in their periodical weight gain. Similarly, the thermal stress treated larvae showed a significant reduction in the length. Our results agree with the findings of Devi and Karuna, (2012), Wongsorn *et al*., (2015) and Rahmathulla & Suresh, 2013). Tanjung *et al*., (2017), noted that, the larval weight and pupal weight considerably reduced with increasing temperatures 34, 38 and 42ºC. A diminution in the body weight of fifth instar larva in response to high temperature was also reported by Lopes *et al*., (2023). Rahmathulla *et al*., (2004) indicated the deleterious effect of temperature shock on the larval growth, especially the decreasing of larval weight under high temperature. In the present observations we can underscore the negative effect of thermal stress on the growth and development of silkworm

5.2. Variation in biological trait

5.2.1. Mortality percentage

Rapid increase in environmental temperature will adversely affect the growth and development of insects (Van *et al*., 2007). Silkworms are highly vulnerable to changing environmental temperature. The survival rate of silkworms decreased along with increasing environmental temperature (Li *et al*., 2018; Mir and Qamar, 2018). Our study observed a high mortality rate in the thermal stress exposed fifth instar larvae in comparison with control. The similar trend reported by Tanjung *et al*., (2017). Sinha and Sanyal, (2013) conducted an experiment at 17ºC, 33ºC, and 43ºC, in which the silkworm larvae can abide at 17ºC and 33ºC but at 43ºC, the larvae tend to die. This is because of the susceptibility of larvae to the high temperature. The mortality rate of larvae increased with high rearing temperature (Khaliq *et al*., 2014). Reduced survival rate of silkworm at high temperatures reported by Chakraborty and Dastidar, (2022). Our result also aligns with the findings of Lopes *et al*., (2023). In our study we noticed that dead larvae in the thermal stress exposed group produced a yellow fluid, it may be due to the inadequacy of larvae to withstand the high temperature and this may lead to the destruction of the body fluid maintenance. A similar observation was made by Tanjung *et al*., (2017).

5.3. Biochemical parameters

Temperature is the primary environmental factor that affects the life of insects, especially their growth, reproduction and their geographical distribution (Worner, 1988; Bale *et al*., 2002). High temperatures are a significant stressor that can drastically alter the physiological activities of insects, notably by triggering a stress response. This thermal stress is a primary cause of the excessive production of ROS, which subsequently leads to oxidative cell injury (Living stone, 2001). A minimal level of ROS is required for the functioning of normal transduction pathways to perform the complex cellular processes (Seo *et al*., 2000). Beyond the normal level of ROS; it causes rigorous damage to the cellular components like lipids, proteins and nucleic acids (Jia *et al*., 2011; Grotto *et al*., 2009). To alleviate the oxidative damage, an antioxidant defense system exists in insects. Normally a homeostasis occurs between the production of reactive oxygen species and activity of antioxidant enzymes (Lopez-Martinez *et al*., 2008). In silkworms thermal stress also exerts variations in all biological processes with biochemical and physiological reactions (Hazel, 1995 and Willmer *et al*., 2004). For understanding the effect of thermal stress on the biochemical parameters such as lipid peroxidation level, total protein concentration, and different antioxidant enzymes such as CAT, SOD, POX, GST, GPX and GR were studied.

5.3.1. Assessment of lipid peroxidation extent

Level of lipid peroxidation quantified by gauging the concentration of MDA. Increased levels of LPO are an indicator of oxidative stress. So, the concentration of MDA is considered as the biochemical indicator of oxidative stress. MDA produced by the oxidation of peroxidized polyunsaturated fatty acids (Del Rio *et al*., 2005). In our study the level of MDA increased in the silk gland, midgut, fat body of larva, pupa and adult of thermal stress exposed group in contrast to control. This indicates the level of lipid peroxidation in the thermal stress exposed group, mediated to the formation of oxidative stress. Lipid peroxidation also discloses the cell injury (Del

Rio *et al*., 2005). The similar observations were reported by Zhang *et al*., (2014); Jia *et al*., (2011); Jena *et al*., (2013) and Makvana *et al*., (2021).

5.3.2. Total protein concentration

The examination of the total protein concentration in the control and experimental group showed variations in different developmental stages of silkworm. The larval silk gland and fat body in the thermal stress exposed group displayed a significant increase in the total protein concentration, on the contrary the midgut tissue of the experimental group showed decreased total protein concentration in the thermal stress exposed group. While exposure to thermal stress resulted in a decreased total protein concentration in the pupae and adult fat body. The increased total protein concentration in the thermal stress exposed tissues alludes to the increased metabolic rate and formation of synthesis of highly conserved new heat shock proteins. This observation is also consistent with the findings of Hightower, (1991); Joy and Gopinathan, (1995); Lokesh, (2018). The decreased protein concentration may be due to the interruption or arrest of the normal level protein synthesis by the revelation of high temperature. This result agrees with the findings of Sreekumar *et al*., (2007). Variations in the protein concentration under thermal stress were also reported by Kumari *et al*., (2020).

5.3.3. Antioxidant enzymes

CAT is one of the principal antioxidant enzymes, responsible for scavenging H_2O_2 (Jena *et al*., 2013), while only high cellular concentrations of CAT be able to remove H_2O_2, whereas low concentrations of CAT is inept to eliminate H_2O_2 (Ahmad *et al*., 1991). In this study activity of CAT in larval silk gland, fat body of pupa and adult displayed a significant increase in thermal stress exposed group in response to control. The increased activity of CAT may be due to the over production of ROS under thermal stress. Apparently, it helps to eliminate the H_2O_2 and prevents the damage caused by oxidative stress. Our findings are in line with those reported by Zhang *et al*., (2014), Lokesh, (2018), Nabizadeh and Kumar, (2011). Although in our study, the midgut and fat body of fifth instar larva showed decreased CAT activity in the experimental group measured against control. This observation aligning with the findings of Nabizadeh and Kumar, (2010), Yang *et al.*, (2010). This may be due to the acute thermal stress 40±2ºC exerted high oxidative stress in the mid gut and fat body. The elevated free radicals may diminish the activity of CAT. A high level of superoxide radicals produced in response to oxidative stress was a reason for the reduced activity of CAT (Dubovskiy *et al*., 2008).

Among the antioxidant enzymes SOD is the most powerful antioxidant (Bafana *et al*., 2011), it transmutes superoxide anion (O_2^-) in to oxygen and hydrogen peroxide (Kashiwagi *et al*., 1997). Our study displayed an increased activity of SOD in the silk gland of fifth instar larva in the test group, on the other hand the midgut of larva, fat body of larva, pupa and adult implicated a reduced SOD activity in the experimental group compared to control. The activity of SOD showed variations in the larval, pupal and adult stages when exposed to thermal stress. This study suggests that increased activity of SOD in the silk gland of an experimental group protected from thermal stress induced oxidative stress. Similar studies were, mentioned by Makwana, *et al*., (2021), Jia *et al*., (2011), Cui *et al*., (2011). The decrease is also reported by Lushchack and Bagnyukova (2005) and Bhawane *et al*., (2013). The decreased SOD activity in the treated samples may be due to increased level of oxidative stress induced by thermal stress and an imbalance between pro-oxidants and antioxidants. Similar observations were reported by Mohankumar and Ramaswamy, (2006).

POX converts H_2O_2 in to water and oxygen (Meng *et al*., 2009). The observed activity of POX was higher in the silk gland of larva treated with temperature shock in contrast to control group. Zhang *et al*., (2014) and Ali *et al*., (2017) previously demonstrated the same trend of results. Moreover, larval midgut and fat body, fat body of pupa and adult showed a decreased activity of POX in the thermal stress exposed group. Jia *et al*., (2011) also observed a similar activity of POX.

The hydroperoxides are the product of lipid peroxidation which is removed from cells by antioxidant enzyme GST (Dubovskiy *et al*., (2008). It is a multifunctional dimeric enzyme; it functions in detoxification, isomerization and intercellular transportation (Zhang *et al*., 2015; Board and Menon, 2013). Activity of GST showed significantly higher in the silk gland, midgut of fifth instar larva and fat body of adult in the heat shock exposed group on comparison with control, conversely a significant reduction showed in the activity of GST in the fat body of larva and pupa. The inhibition of GST activity may be due to the alteration of antioxidant defense mechanism ((Dong *et* *al*., 2024). Elevated levels of GST activity also observed by Jia *et al*., (2011), Zhang *et al*., (2015), Ali *et al*., (2017). Increased activity of GST indicates protection of silkworms from oxidative damage by removing the hydroperoxides formed by the by product of lipid peroxidation resulting from intense heat shock during the investigation phase.

GPX is an antioxidant responsible for detoxify H_2O_2 and catalyses the reduction of lipid peroxide and other organic peroxide with the addition of supplementary substrate GSH (Jena *et al*., 2013; Halliwell and Gutteridge, 2001). An augmented activity of GPX showed in the silk gland and fat body of larva. This observation aligns with the work of Altuntas *et al*., (2020), Mutusamy and Rajakumar (2016) and Yuan *et al*., (2013). A reduction in the action of GPX was observed in the midgut, pupal fat body and adult fat body of heat shock exposed group compared to control. Decreased activity of GPX likely attributed to the increased synthesis of ROS during heat shock. A similar observation was made by Muthangi, (2020).

The antioxidant enzyme Glutathione reductase (GR) revives the reduced glutathione (GSH) from oxidized glutathione (GSSH). It acts as a ROS scavenger and also a substrate for additional enzymes (Jena *et al*., 2013; Halliwell and Gutteridge, 2001). GR activity significantly higher in the larval silk gland, midgut and fat body, fat body of pupa and adult in the thermal stress exposed group than control. Increased activity of glutathione reductase in the thermal stress exposed group indicates the conversion of oxidized glutathione to reduced glutathione. Reduced glutathione plays a prominent role in the neutralization of free radicals and protecting cells from oxidative damage (Summers and Felton, 1994). Our observations align with the findings of Mutusamy and Rajakumar, (2016).

5.4. Gene expression

5.4.1. Transcriptional co-activator genes

Transcriptional co-activators have a major role in gene transcription and expression (Li *et al*., 2009). Investigation of the consequences of temperature exposure on the expression profile of transcriptional co-activator genes showed variation in different developmental stages. The BmYki gene takes part in multiple key administrative roles in the hippo signaling pathway. BmYki genes and alternative splicing isoforms participated in the organ growth and development, cell proliferation and apoptosis (Zeng *et al*., 2017, Zeng *et al*., 2018).

The transcriptional co-activator BmYki gene expression is declined in the experimental group of midgut, fat body tissues of fifth instar larva and adult. Transient high temperature treatment (HT 48 h) of midgut samples reported the same trend of gene expression in silkworm *Bombyx mori* (Sun *et al*., 2017). The Hippo signaling pathway controls the cell size and cell cycle (Yu *et al*., 2015). Generally during the fifth instar, the larvae actively feed on the leaves to gratify the demands of augmented hunger, an account of this, the midgut epithelium and midgut hurriedly grow and develop (Sun *et al*., 2017). In this work, the down regulation of BmYki gene in response to thermal stress may indicate repression of the hippo signaling pathway in midgut, fat body of larva and adult and also may indicate the role of BmYki in organ growth and development. In our study, the treated pupal fat body showed a slightly upregulated expression of BmYki in contrast to control. This may indicate the activation of hipposignalling pathway in pupal stage.

On quantitative study of expression pattern of BmYki during different developmental stages revealed that, it was highly expressed in the testis, ovary, head and trachea, compared to this tissues, expression normally dominated in the silk gland of day three larvae of fifth instar (Zeng *et al*., 2017). Yki location is mainly cytoplasmic in both drosophila and silkworm (Goulev *et al*., 2008; Zeng *et al*., 2017). Zeng *et al*., (2017) also suggest that over expression of BmYki down regulated the silk protein synthesis genes and also promoted the expression of genes related with cell proliferation and apoptosis. In the present study, in comparison with the control, the silk gland of the experimental group revealed a significant upsurge in the expression of BmYki. This may be due to the regulation of the repair of damaged silk gland, by the formation of oxidative stress after subsequent exposure to heat shock, may be due to the activation of hippo signaling pathway.

MBF1 is a transcriptional co-activator that normally works in gene transcription (Li *et al*., 2009). Besides, it also functions as a stress response element in different eukaryotic organisms. MBF1 proteins, like heat shock factors, have undergone extensive conservation in a variety of species, including humans, flies, yeast and plants (Von Koskull-Doring *et al*., 2007: Takemaru *et al*., 1997; Takemaru *et al*., 1998; Brendel *et al*., 2002; Liu *et al*., 2003). In the current study the thermal stress induces an up- regulated expression of MBF1 gene in midgut, fat body of fifth instar larvae and fat body of pupae in comparison with control. Upon ethanol exposure to drosophila showed an up regulated expression of mbf1 gene to regulate oxidative stress (Awofala *et al*., 2012). Jindra *et al*., (2004) suggested that in drosophila, MBF1 is also responsible for the maintenance of redox dependent AP1 activity in oxidative stress conditions. A similar result was reported in a previous study which found that, an upregulated expression of MBF1 in the model organism *Pyropia yezoensis* during elevated temperature (Sun *et al*., 2015). Chu *et al*., (2015) also reported that fungal entomopathogen *Beauveria bassiana* under oxidative stress showed an increased expression of BbMBF1.On comparison with these previous reports, the present study revealed that MBF1 may play a major role in heat stress and oxidative stress pathways in silkworm *Bombyx mori.* On the contrary the vertebrate Maraena whitefish (*Coregonus maraena*) subjected to gradual and acute temperature showed a down regulated expression of EDF1 (alias MBF1) in both spleen and kidney (Rebl *et al*., 2018) , the similar trend was showed in our results, when adult moth exposed to high temperature. The MBF-1 doesn't play a role in thermal stress in the adult. In this study the expression of MBF1 in response to thermal stress may vary up on the tissue and stage specific level of development.

5.4.2. Pro-apoptotic genes

Apoptosis is a programmed cell death mechanism. It caused by various kinds of physiological as well as developmental incitements (Selvakumar *et al*., 2013), although apoptosis is also prompted by elevated temperature (Jin *et al*., 2007; Hsu *et al*., 2011). It is the process of deletion of unnecessary harmful cells undergoing several complex events (Selvakumar *et al*., 2013). Apoptosis has a profound effect on tissue homeostasis, host defense mechanism, development in multicellular organisms (Steller, 1995; White, 1996), and resistance to environmental stress (Fulda and Vucic, 2012). Apoptosis is an essential feature of silkworm immunity (Yang *et al*., 2018).

Pro-apoptotic genes are genes which promote apoptosis. There are two important apoptotic pathways intrinsic and extrinsic. A previous report gives an idea about presence of apoptotic genes and pathways in silkworm *Bombyx mori* (Zhang *et al*., 2010). This investigation examines the sequel of heat exposure persuaded oxidative stress in the expression of pro-apoptotic genes such as BmApaf-1 and BmICE-2. BmApaf-1 is one of the important pro-apoptotic gene present in silkworm, which promotes mitochondrial-apoptotic pathway by H_2O_2 induced oxidative stress in silkworm *Bombyx mori* ovarian cells (Chen *et al*., 2015). BmApaf-1 also serves a significant role in metamorphosis. An azaserine induced oxidative stress also leads to the increased expression of BmApaf-1(Muthangi, 2020). The present study displayed regulated expression of BmApaf-1 against thermal stress induced oxidative stress in larval silk gland and fat body, and fat body of pupa and adult. This may indicate the activation of mitochondrial pathway in larval pupal and adult developmental stages of silkworm in contrast to thermal stress induced oxidative stress. Previous report also supports up-regulated expression of Apaf-1 gene under thermal stress (Zhang *et al*., 2014; Zhang *et al*., 2015; Zhang *et al*., 2017). Our results revealed that, may be the activation of mitochondrial apoptotic pathway under thermal stress helps to remove the damaged cells and protect the silkworms from thermal stress induced injury. And may it have a special role in tissue homeostasis, growth and development and host defense under thermal stress conditions. In our study the larval midgut implicated a down regulated fold difference of gene expression in the experimental group in relation to control. This may be due to the inactivation of mitochondrial apoptotic pathway in midgut under thermal stress, and may take place in any other programmed cell death process other than apoptosis to alleviate the oxidative stress.

The caspase family constituent BmICE-2 is a newly identified pro-apoptotic gene found in silkworm (Yi *et al*., 2014). Caspase family component ICE (Interleukin 1β converting enzyme) was detected in mammals, and they act as initiators in the process of apoptosis. In *Caenorhabditis elegans* ICE identified as a CED-3 like protein (Yuan *et al*., 1993). On the other hand, in Lepidopteran insects the pro-death factor ICE identified in mid guts of *Heliothis virescens* (Parthasarathy and Pilli, 2007). In silkworms there are three ICE homologs, ICE, ICE-2 and ICE-5 (Zhang *et al*., 2010). BmICE-2 displayed a caspase9 proteolytic activity, and promotes apoptosis in BmN-SWU1cells and BmE-SWU1 cells (Yi *et al*., 2014).

In this analysis, the fold expression of BmICE-2 gene was up-regulated in the pupal and adult fat body in the thermal stress exposed group. Sun *et al*., (2008) observed a similar trend of BmICE-2 expression in silkworm *Bombyx mori* ovarian cells under oxidative stress induced by H_2O_2 and ultraviolet radiation. One of the ICE homolog BmICE exhibited an upregulated trend of expression when BmN-SWU1 cells exposed to H_2O_2, reported by Chen *et al*., (2015). In our study the fifth instar larval stage, tissues such as silk gland, midgut and fat body showed a down regulated expression, it may be due to the inhibition of BmICE-2 by any other factors under thermal stress. Therefore, further studies have to be conducted to determine the cause of down regulated expression of BmICE-2 under thermal stress.

Both BmApaf-1 and BmICE-2 are pro-apoptotic genes presented in silkworm. In this investigation both genes are detected in all developmental stages under thermal stress, but the expression pattern varies in different tissues and developmental stages of silkworm under thermal stress. Heat stress can trigger apoptosis and eventually impact the cell proliferation and differentiation (Gu *et al*., 2015; Zhou *et al*., 2020). Our results suggest that upregulated expression of BmApaf-1gene subsequent to elevated temperature in larval, pupal and adult developmental stages may be an indication of actuation of mitochondrial apoptotic pathway. The expression of BmICE-2 gene in thermal stress exposed group of pupa and adult also may be a portend of activation of apoptotic pathways.

5.4.3. Inflammatory response genes

Silkworm *Bombyx mori* comprises an innate immune system instead of deficiting adaptive immunity (Cheng *et al*., 2008). Fat body and haemocytes is the major tissue and cell accountable for the insect's innate immunity (Lu, 2008). Insect's fat body functionally resembles the mammalian liver and it generates ROS, antimicrobial peptides (AMPs) and other humoral response factors (Aggrawal and Silverman, 2007). Silkworm immunity is accomplished by the combination of cellular immunity, humoral immunity and cell apoptosis (Yang *et al*., 2018).

IL-6 is an inflammatory response gene. It is a multifunctional cytokine that encompasses various obligations in many physiological processes such as "acute phase response, immune response, host defense, inflammation haematopoiesis and cellular growth" (Kishimoto, 2006). The initiation and resolving inflammation are the important role of IL-6, when the deregulation of IL-6 leads to the generation of chronic inflammatory and autoimmune diseases. Moreover IL-6 is a key factor in the pathogenesis of numerous human malignancies (Ataie-Kachoie *et al*., 2014). IL-6 produced by different kinds of cells, they are "T" and "B" lymphocytes, fibroblasts, monocytes, keratinocytes, mesanglial and endothelial cells and tumour cells (Ataie-Kachoie *et al*., 2013, Ataie-Kachoie *et al*., 2014).

Authentic reports are not available to interpret the role of IL-6 and TNFSF5 under thermal stress or any other abiotic stress in silkworm *Bombyx mori*. We studied the impact of elevated temperature on the expression analysis of IL-6 and TNFSF5 in silkworm. In our study the oxidative stress induced by temperature exposures results in changes in the expression pattern of IL 6 gene in silkworm during different developmental stages. The fifth instar larval fat body, pupal fat body, and pupal haemolymph of thermal stress exposed group showed a significant upregulation of IL-6 gene. Previous report showed that chemical exposure induces oxidative stress and in turn resulted in the upregulated expression of IL-6 (Fang *et al*., 2021). Thermal stress and H_2O_2 induced oxidative stress displayed an elevated expression of cytokines in insect *Mythimna seperata* reported by Matsumura *et al*., (2018). The finding of our results correlated with Wang *et al*., (2019), Rawash *et al*., (2022), Butterweck *et al*., (2003), Li *et al*., (2023), Al- Zghoul, *et al*., (2019), Park *et al*., (2015) . On the other hand, in our study, the fifth instar larval hemolymph and adult fat body displayed a considerable down regulation of IL-6 in the experimental group. The down regulated expression of IL-6 in tissue samples of treated groups may reveal the absence of inflammation especially in the fat body of larvae and adults. Our findings indicated that further studies are necessary to establish the down regulation of IL-6 in the thermal stress exposed group of larval haemolymph and adult fat body. During stress conditions or any injury, the IL-6 possesses special roles in tissue protection, healing and regeneration of damaged tissues (Streetz *et al*., 2000; Welc *et al*., 2013a). So, in silkworm the IL-6 may act as a tissue protective and wound healing agent and may also play a special role in regeneration of damaged tissues during the induction of heat shock.

TNFSF5 is also an inflammatory response gene present in silkworm. The current investigation the larval stage tissues of fat body, haemolymph and pupal fat body of treated group implicated a down regulated expression of TNFSF5 gene in comparison with control. While in the pupal haemolymph and adult fat body displayed an upregulated expression. The upregulated expression of treated group showed a similarity in the gene expression reported by Fang *et al*., (2021) and Park *et al*., (2015). The upregulated trend of expression of TNF Superfamily receptors under heat shock were also reported by Wang *et al*., (2023). The down regulated expression of TNFSF5 in the treated group of larval tissues may be that it doesn't play a role in inflammation in the larval stage. The upregulated expression of TNFSF5 in silkworm pupal and adult stages indicates the incidence of inflammation in response to heat exposure.

Thermal stress exerts oxidative damage to the body of bivoltine and polyvoltine silkworm strains when exposed to 35ºC and 40ºC (Makwana *et al*., 2021). This study revealed that the significant upregulation of IL-6 and TNFSF5 gene in silkworm exposed to high temperature, indicating the occurrence of inflammatory responses in larva, pupa and adult developmental stages. The inflammatory response genes may play a role in host defense mechanisms when silkworms exposed to high temperatures, they were easy to susceptible for pathogen infections. However, the impact of heat shock on gene expression of inflammatory response genes like IL-6 and TNFSF5 in silkworm is still unknown. This will be a new insight into the information regarding the effect of thermal stress on inflammatory response genes in silkworm.

5.4.4. Caspase 3 gene

Caspase-3 gene is an apoptotic marker gene (Jorgensen *et al*., 2017). In the apoptotic pathway the activated caspase-9 triggers the activation of caspase3 and eventually the cell undergoes apoptosis (Beere, 2004). From the silkworm genome 52 apoptotic related genes have been identified, in which there are five caspase family members included (Zhang *et al*., 2010). It is yet unclear how they contribute to programmed cell death. So, in this study we examined the expression analysis of Caspase-3 gene under induction of heat shock.

In our study the expression of caspase 3 displayed a significantly increased trend of fold difference of gene expression in silk gland, and fat body of larvae, pupae and adults of treated group. While in experimental mid gut showed a significantly down regulated fold expression. Normally the posterior silk gland of silkworm during spinning period showed an upregulated trend of caspase3 gene which indicates the occurrence of apoptosis of posterior silk gland (Hu *et al*., 2018). Dilapidation of chromosomal DNA and initiation of caspase3 activation frequently accompany apoptosis (Franzetti *et al*., 2012). In our study the thermal stress induced oxidative stress may exert DNA damage and it may lead to activation of caspase3 expression. The upregulated gene expression in the experimental samples indicates the activation of apoptotic pathways in larval, pupal and adult fat body silkworm. The reduced expression of caspase3 in the midgut, suggests the non-occurrence of apoptosis under temperature stress. Similar trends of findings were presented by Yang *et al*., (2020); and Cheng *et al*., (2015). They reported the increased activity of caspase 3 and upregulated expression of different pro-apoptotic caspases in silkworm BmE cells under treatment of UV radiation. Activation of caspase 3 activity in silkworm BmN cells was reported by Ye *et al*., (2023).

5.4.5. HSP 70

The heat shock protein Hsp 70 is a molecular chaperon that has constitutive and inducible forms which have a specific activity in stress response (Nollen *et al*., 1999; Dahlgaard *et al*., 1998).

The larval tissues such as silk gland, midgut and fat body showed an increased expression of Hsp 70 in thermal stress exposed group compared to control. In the pupal and adult tissues implicated an upregulated expression of Hsp 70 gene in the experimental group. In these observations we can interpret that Hsp 70 performs a major role against thermal stress. Similar observations were obtained in silkworm *Bombyx mori* at high temperatures of 38ºC and 42ºC also indicating that Hsp 70 show a critical role in stress response (Velu *et al*., 2008). Similar trend of results were reported by Zhang *et al*., (2017), Cheng *et al*., (2015), Snutch *et al*., (1988), Li *et al*., (2014), Cui *et al*., (2011). Hsp70 also have an antioxidant activity (Creagh *et al*., 2000; Sreedhar and Csermely, 2004). The upregulated expression of Hsp70 may assists to reduce the oxidative stress. Moreover, synthesis of Hsp 70 obstructs the exorbitant apoptosis (Cui *et al*., 2011; Samali and Orrenius 1998; Carpenter and Hofmann, 2000). So, if the thermal stress induced apoptosis will follow an uncontrolled or excessive stage and it will be prevented by Hsp 70.

5.5. Histopathology of larval midgut

The digestive system of silkworm *Bombyx mori* consists of three parts, foregut midgut and hindgut (Judy and Gilbert, 1969). The midgut originates from the endoderm cells and which is the major part for the ingestion and transportation of food, digestion of food, nutrient absorption and maintenance of ionic balance (Levy *et al*., 2004). The midgut which constitutes the largest part of the alimentary canal, it consists of a peritrophic membrane, columnar epithelial cells, goblet cells and regenerative cells. The peritrophic membrane protects the epithelial cells from hard food particles by encircling the food particles (Mathur, 1973). Columnar epithelial cells are concerned with the function of digestion and absorption although goblet cells carry a role in ionic balance (Billingsly and Lehane, 1996). This investigation sought to examine the impact of thermal stress on the structure and gut histology of fifth instar larvae.

In our study the control midgut showed an intact arrangement of midgut cells while thermal stress exposed midgut showed alterations in the structural and histological organization of midgut. After the exposure of heat shock goblet cells become enlarged and blended with epithelial cells and form an irregular arrangement of cells. This observation points out the cellular stress and damage caused by thermal stress induced oxidative stress. The akin observations were also founded by Sun *et al*., (2022) and Fast and Angus (1965). Silkworms are highly sensitive to environmental stress. High temperature is an important abiotic factor affecting the growth and development of silkworms. The histopathological evaluation of the midgut revealed that the temperature stress affected the midgut structural orientation. The intestinal metabolic processes are controlled by the joint activity of host genes and microbial genes (Broderick and Lemaitre, 2012; Dayama *et al*., 2020). The heat stress may also affect the intestinal microbe composition and it will affect the digestion of the silkworm. This eventually affects the growth and development of silkworms.

5.6. Quantity and quality assessment of cocoon

When looking at the current global scenario, climate change and global warming are one of the major global environmental problems, especially the extreme weather event such as soaring temperature (Frame *et al*., 2020). Climate change is one of the major reasons for declining insect populations (Dar and Jamal, 2021b). When we consider these situations, a study of the sequel of temperature exposures on the life of silkworm is very necessary because they are very thermal sensitive organism among insects.

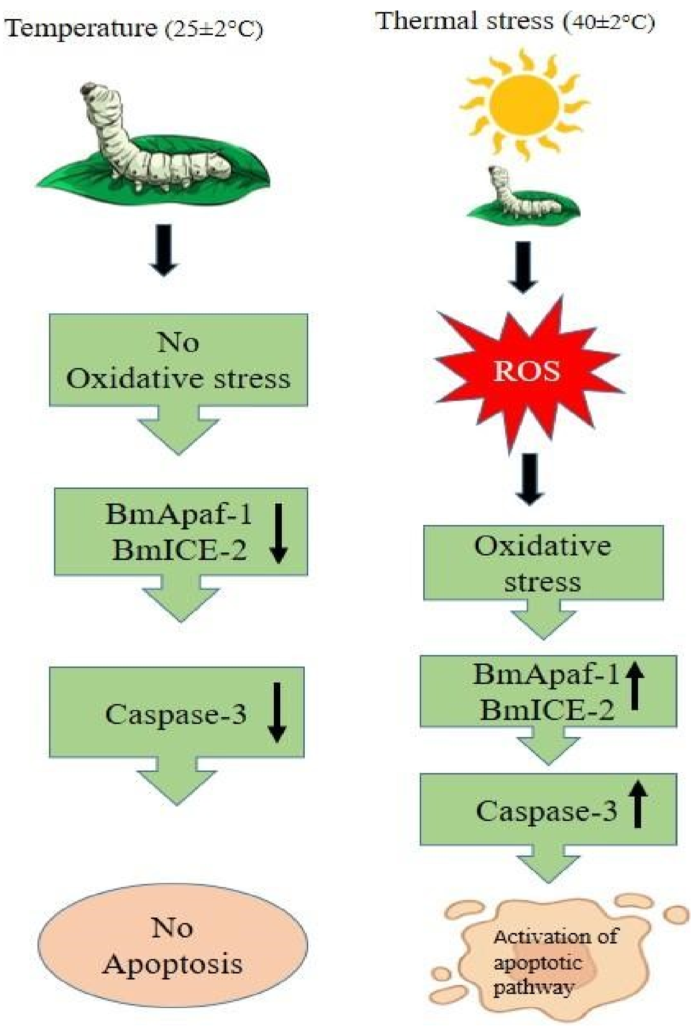
Silkworm *Bombyx mori* is the major insect cultured for silk production, the environmental conditions, growth of the larvae, and health of the larvae are the major factors affecting the cocoon productivity and quality in sericulture (Kumar *et al*., 2001). The focal objective of sericulture is the production of good quality cocoons. Optimum temperature 25-28ºC and relative humidity 60-75% is the ideal environmental conditions required for the larval growth and development, adult emergence and bulk production of silk. Elevated temperature and RH directly affect the process of spinning and characteristics of sericin, and it finally resulted in changes in the cocoon properties like strength, rigidity and coloration (Offord *et al*., 2016; Ramachandra *et al*., 2001).

In this study we focused on the effect of thermal stress on the different parameters of cocoon like length, width, weight, shell weight, shell percentage, filament length and weight, Denier, and reelability percentage of cocoon. These are the most important cocoon parameters that allude to the quality and quantity of cocoons, and examination of these parameters under thermal stress conditions gives valuable information regarding the upshot of heat stress on the production of cocoons. In our observations all the cocoon parameters were remarkably decreased in the thermal stress exposed group with respect to control. Our observations are agreement with the results of Lopes *et al*., (2023), they reared the fifth instar silkworms under 26ºC and 34ºC, the cocoon weight drastically reduced at high temperature exposed group compared to control. Khan *et al*., (2014) observed that, increasing environmental temperature results in the decreased cocoon weight in response to ambient normal temperature 25ºC. Tanjung *et al*., (2017) treated the silkworm larvae at varying temperatures like 34, 38 and 42ºC with response to an ambient temperature, their findings displayed a reduced larval growth and development, which leads to the reduction in the cocoon productivity. The single cocoon weight and shell weight was considerably, decreased in the thermal stress exposed groups in contrast to ambient temperature.

An increase in rearing temperature can affect the quantitative and qualitative traits of cocoon such as cocoon weight, shell weight, shell ratio, filament length, filament weight, and denier of the cocoon. The adverse effects of temperature on these traits are reported by Nabizadeh and Kumar, (2010). The findings of Rahmathulla *et al*., (2004) also revealed the negative impact of thermal stress on the cocoon weight and shell weight. These two cocoon characters are reduced in high temperatures in comparison to the control. They also noticed that an increased consumption of mulberry leaves was recorded in the control group; on the other hand, there was a reduction in the intake of mulberry leaves in the high temperature exposed group. Digestion rate also diminished in the thermal stress exposed group in relation to the control. Ingesta and digesta significantly affected the quality of the cocoon. Our study also displayed a momentous reduction in the quality and quantity traits of cocoons. This implies that the treatment of high temperature directly influences the feeding of mulberry leaves, it subsequently affects the growth and development of larva and ultimately the production of good quality cocoon was decreased with response to high temperature.

Elevated temperature above 30ºC and temperature below 20ºC, the silkworms reared under these conditions are easily susceptible to diseases; this is another factor for reducing cocoon production. They also implicated that nearly all the quantitative and qualitative traits, the reelability percentage and yield are the most impacted characters to the adverse environmental conditions (Rahmathulla, 2012). In the current global scenario, management of high temperature is a major task to improve the quality and quantity of cocoons.

Figure: 5.1. Schematic depiction of impact of thermal stress on the activation of apoptotic pathway in silkworm *Bombyx mori.*



CONCLUSION

6. CONCLUSION

In the present study we probed the upshot of heat shock on the biochemical, physiological as well as molecular responses of various silkworm developmental stages. We primarily focused on the impact of heat shock on the expression of transcriptional co-activators, pro-apoptotic markers and inflammatory response genes. Our results indicated that thermal stress exerts alterations in the expression of these genes in different developmental stages of silkworm in a tissue specific manner. Also thermal stress negatively affected the growth and development of silkworm *Bombyx mori.* Following are the conclusions of the findings.

- Examination of the morphometric parameters of different developmental stages of silkworm revealed that thermal stress is harmfully affecting the larval pupal and adult phase's development.
- Analysis of the fifth instar larval mortality percentage, revealed a sudden decline in survivability in the experimental group, strongly indicating the adverse effects associated with thermal stress.
- Conspicuously thermal stress is a reason for the generation of ROS which leads to the formation of oxidative stress in the cells and tissues, evidenced by the increased MDA concentration in all the experimental tissues.
- Thermal stress exposure implies a diverse trend of total protein concentration in different experimental tissues. Increased total protein concentration in the thermal stress exposed group may be due to the synthesis of new Hsp proteins during high metabolism to withstand the thermal stress. On the other hand some experimental tissues inhibited the normal protein synthesis due to thermal stress induced oxidative stress.
- Treatment with high temperature resulted in alternations in the biochemical activities of different tissues of various developmental stages of silkworm. Activation of antioxidant enzymes in the silk gland exhibits the performance of a defense system to scavenge the ROS formed during thermal stress. All other examined tissues evidenced alterations in the activity of antioxidant enzymes, most of the tissues showed inactive defense systems. This may be due to the increased

generation of ROS under thermal stress. Excess production of ROS may exert an imbalance between pro-oxidants and antioxidants; this may inhibit the antioxidant defense system under oxidative stress conditions.

- Our analysis established the repercussions of thermal stress on the expression of transcriptional co-activators in different tissues of different developmental stages. The upregulated expression of MBF1 gene protects silkworms from oxidative damage in larval and pupal stages.
- The gene BmYki played a prominent role in organ growth and development under thermal stress. Normally over-expression of BmYki down regulated the silk protein synthesis genes and it also promoted cell proliferation and apoptosis. In our work it is evident that the upregulated expression of pro-apoptotic BmApaf-1 gene and apoptotic marker gene caspase 3 in the silk gland of the thermal exposed group. The incidence of apoptosis facilitates the removal of damaged silk gland cells and protects the organism from thermal stress. Apparently the upregulated expression of BmYki and BmApaf-1 genes during thermal stress results in the production of low quality and quantity of cocoons.
- The upregulated expression of BmApaf-1 and BmICE-2 genes in diverse developmental phases of silkworm indicates the activation of apoptotic pathways in response to thermal stress induced oxidative stress.
- The present enquiry showed the increased expression of IL-6 and TNFSF5 in silkworm developmental stages implies the occurrence of inflammation during thermal stress.
- Apoptotic marker gene caspase 3 upregulated in various developmental phases of silkworm suggest the occurrence of apoptotic process.
- During thermal stress, Hsp70 gene upregulated in all developmental stages, suggesting that it confers protection against oxidative damage. Furthermore, its upregulation may inhibit excessive apoptosis under the influence of thermal stress.
- Our findings underscore the detrimental effect of thermal stress on the quantity and quality of cocoons such as length, width, shell weight, shell ratio, filament length, filament weight, denier and reelability percentage of cocoon. Undoubtedly we can

affirm that thermal stress exerts a negative impact on the growth and development of silkworms and quality and quantity of cocoons.

- These findings clearly state the biological processes associated with temperature response mechanisms of silkworm, offering valuable insights for optimizing climate control in rearing facilities and for screening more thermo tolerant strains.
- The observations of this study revealed that thermal stress induced oxidative stress induces the expression of apoptotic related genes and stress response genes. This may be due to the activation of apoptotic pathways under thermal stress. This study contributes valuable insights in to the stress response mechanism of silkworm in biochemical and molecular levels. It is imperative to examine the detailed mechanisms and pathways related to apoptosis under the influence of thermal stress in silkworm.

RECOMMENDATION

7. RECOMMENDATION

Silkworm is a thermal sensitive organism; it is widely used as a paradigmatic organism in different research areas associated with human ailments, selection of antimicrobial agents, environmental safety monitoring, and antitumor studies. The current research work demonstrated the impact of thermal stress on the gene expression of transcriptional co-activators, apoptotic related genes and inflammatory response genes. Regarding the current study, we would like to suggest the following recommendations for the future investigations.

- Further research is required to elucidate the apoptotic pathways activated at various temperatures and it is necessary to quantify the rate of apoptosis induced by temperature stress.
- Moreover it is important to study the genes related to stress response and mechanisms behind the control of expression of stress response genes.
- Accomplishing comparative studies with different stressors adversely affecting sericulture such as temperature, humidity and pathogen infections, and also combination of these stressors to find out the information regarding varying cell death pathways.
- Further research should be undertaken to determine the precise inflammatory and anti-inflammatory response pathways during thermal stress. Silkworm haemocytes have anti-inflammatory effects. Studies of inflammatory and anti-inflammatory pathways may be beneficial for medicinal insect research.
- Research is needed to scrutinize the balance between pro-oxidants and antioxidants produced during thermal stress. The excessive production of pro- oxidants under oxidative stress conditions may inhibit the activity of antioxidants.
- Although study is necessary to investigate the quantity, biological and mechanical properties of silk biomaterials such as sericin and fibroin on the influence of thermal stress induced apoptosis, because now they have a special attention in the pharmaceutical industry and has wide applications in nanotechnology, tissue engineering, cosmetics etc.

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List of publications

- **Peer Reviewed International Journal Paper**

1. Sajini K.P. and C.V. Sreeranjitkumar (2022). Expression of BmApaf-1 and BmDredd Genes and Antioxidant Responses of Silkworm *(Bombyx mori* L.)Exposed to Thermal Stress. *Research Journal of Agricultural Sciences An International Journal,* 13(5): 1628–1634, 0976-1675, Centre for Advanced Research in Agricultural Sciences.

List of papers presented at conferences

1. **International conferences**

i. Sajini, K. P., and Sreeranjitkumar, .C.V. Presented a paper entitled "The effect of thermal stress in the expresssion of BmCaspase-1 and BmSpatzle 4 and antioxidant system in the silk gland of *Bombyx mori".* International conference on Recent Advances in Biosciences and Technology (RABT 2021) held at St. Mary's college , Thrissur, Kerala, on 7thDecember 2021.

ii. Sajini K.P., and Sreeranjit Kumar, C. V. Presented a paper entitled "Alternations in the expression profile of immune response genes in silk gland of silkworm *Bombyx mori* L.in response to thermal stress". International Conference on recent Advances in Animal Biology and Conservation (ICRABC 2023) held at University College, Thiruvananthapuram, Kerala, India. December 19th – 21st 2023.

2. **National Conference**

iii. Sajini, K.P., and Sreeranjit Kumar, C.V. Presented a paper entitled "Effect of thermal stress on antioxidant defense system and fluctuations in the gene expression of silk gland of fifth instar larvae, *Bombyx mori L.* Three Day National Seminar on Biodiversity for Sustainable Future held at Sree Neelakanda Govt. Sanskrit College, Pattambi, Palakkad, Kerala, India. November 14th to 16th 2023.

Expression of BmApaf-1 and BmDredd Genes and Antioxidant Responses of Silkworm (Bombyx mori L.) Exposed to Thermal Stress

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Expression of BmApaf-1 and BmDredd Genes and Antioxidant Responses of Silkworm (*Bombyx mori* L.) Exposed to Thermal Stress

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A B S T R A C T

High temperature is one of the major environmental stresses, which is an important reason for altering the physiological responses such as surplus production of reactive oxygen species and consequent oxidative damage among animals especially in insects. This ROS could stimulate intrinsic as well as extrinsic pathways of apoptosis. The present study envisages appraising the impact of thermal stress on the expression of apoptotic related genes BmApaf-1 and BmDredd and the response of antioxidant enzymes (SOD, CAT, POD, GPX, GR, GST) in the silk gland of silkworm. For this study the fifth instar larvae grouped in to control (28±1ºC) and heat exposed group (40±2ºC) and analyzed the expression of BmApaf-1 and BmDredd mRNA by real time PCR using Actin as the internal control. Based on the results we observed a significant 1.7 fold upregulation of BmApaf-1 and 1.5 fold upregulation of BmDredd genes under thermal stress and the antioxidant enzymes showed increased activity against thermal stress to alleviate the consequent oxidative stress.

***Key words*:** Silkworm, Thermal stress, Antioxidant enzymes, BmApaf-1, BmDredd

Silkworms are economically important insects that are thermally sensitive, rearing at agreeable temperatures between 25-28 °C. Temperature, humidity and photoperiod fluctuations are all important factors in the growth and development and production of high-quality cocoons [1]. Elevated temperature has a direct impact on cell organizations, metabolic processes [2] and physiological activities of silkworm [3]. Normally the endogenous oxidative metabolism as well as from different exogenous sources results the formation of ROS, which consists of partly reduced metabolites of oxygen such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals [4-5].

Although ROS does have a range of functions, for example an ideal level of intracellular ROS needed for the mechanism of cellular homeostasis [6], where as a slight amount of ROS render as a signaling molecule in the survival pathways [7]. Excessive production of reactive oxygen species (ROS) can cause oxidative stress [8]. The status of oxidative stress is defined as the disparity between the formation of ROS and the regulation of antioxidant defense system [9].

Antioxidant enzymes and non-enzymatic antioxidants are used to counteract the generation of high amounts of ROS [10]. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), are included in the category of enzymatic antioxidants [11]. Whereas ascorbic acid, vitamin E and reduced glutathione (GSH) are non-enzymatic antioxidants [12]. Each enzyme plays their own role to combat oxidative stress. Superoxide anion can be converted to molecular oxygen and hydrogen peroxide by SOD [11], although the catalytic enzyme CAT [13] and POX [14] convert hydrogen peroxide to molecular oxygen and water. GPX engaged in the detoxification of H_2O_2 as well as reduction of organic hydroperoxides and lipid peroxides, these actions are facilitated by reduced glutathione (GSH,) which functions as a hydrogen donor. The enzyme glutathione reductase liable for the reversion of reduced glutathione (GSH) from oxidized glutathione (GSSG) [11]. The hydroperoxides formed by the byproduct of lipid peroxidation, can be removed by the enzyme GST. The activity of antioxidants may not be enough to control the excessive ROS produced and it will lead to oxidative stress [15].

The stress induced cells become apoptotic or necrotic, due to the structural changes occurred especially in the lipids, proteins and nucleic acids**.** These cellular injuries and the intensity of lipid peroxidation determine the depth of oxidative stress [16]. Unwanted and potentially perilous cells are being eliminated by the physiological process called apoptosis [17]. Apoptosis plays different roles such as tissue homeostasis [18], regulation of development and organ differentiation process, and confrontation against environmental stress [19]. It has two important pathways, intrinsic and extrinsic [20]. ROS shows

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important roles in extrinsic [21] as well as intrinsic pathways [22]. Intrinsic pathway also known as mitochondrial apoptotic pathway [23], triggered by intracellular signals and extrinsic pathway activated by the transmembrane receptor mediated interactions [24]. In intrinsic pathway, mitochondria release cytochrome C makes an apoptosome complex with Apaf-1 and ATP, which activates caspase-9. The activated caspase-9, activate caspase-3 and commence apoptotic process [23]. In the extrinsic pathway, plasma membrane carrying cell death receptors and that have been activated by the binding of extracellular ligands [25] and finally activates the effector caspases [26].

BmApaf- 1(*Bombyx mori* apoptotic peptidase activating factor 1) is similar to mammalian Apaf-1 [27-28] and BmDredd (*Bombyx mori* death related ced-3/Nedd2-like protein) is an initiator caspase homologue [29] shows high similarity with mammalian initiator caspases [30]. Normally insect larval specific tissues are detached during programmed cell death mechanism [31]. In silkworm silk gland is the silk producing larval specific organ [32], which can be decadent quickly following the spinning stage [31]. The present study was conducted to find out the effect of thermal stress in the activity of antioxidant enzymes and the expression of BmApaf-1 and BmDredd genes in the silk gland of silkworm.

MATERIALS AND METHODS

Experimental animals and protocol

Bombyx mori larvae were procured from National Silkworm Seed Organization, Central Silk Board, Pallatheri, Palakkad, Kerala. The rearing was carried out according to the method of [33]. The larvae were reared on an appropriate temperature 25±1 °C and 70-85% relative humidity. They were fed with fresh mulberry leaves *ad libitum.*

In the fifth instar stage the larvae were grouped into control group and test group. The test group larvae were treated with 40±2 °C temperature in 1 hour per/day during the fifth instar stage. The silk gland of fifth instar larvae were selected as the experimental tissue and it was dissected for examining the activity of antioxidant enzymes and gene expression pattern.

Determination of oxidative stress markers

Tissue homogenization was done with an ice-cold PBS buffer of 7. 4 pH, centrifugation at 10000 RPM for 30 min at – 4 °C. After centrifugation the supernatant was kept in pre-chilled eppendorf tubes at -20 °C, used for further studies. The quantification of protein was determined by using Lowry's method [34] in which bovine serum albumin (fraction v, sigma) was taken as standard. The quantity of lipid peroxidation was determined by the method of [35]. 1.0 ml of the tissue homogenate of silk gland was mixed with a mixture of TBA-TCA and HCl reagent. Then the samples were kept in a boiling water bath for 15 minutes and after cooling the samples, then they were centrifuged for up to 10 minutes. The supernatant was used for the estimation of various stress markers. Concentration of lipid peroxidation expressed in terms of µM MDA produced /mg protein.

Catalase enzyme

The Luck [36] was used to determine catalase enzyme activity by drop in absorbance induced by H_2O_2 breakdown at 240 nm, which results in the formation of water and oxygen. Enzyme activity expressed in terms of 1 µM of H_2O_2 consumed /min/mg protein.

SOD enzyme activity

The approach of Marklund and Marklund [37] could be used to determine SOD activity at 420 nm. This approach is based on the amount of enzyme needed to block the autoxidation of pyrogallol by 50%. The activity expressed in units/ml.

Peroxidase enzyme activity

The activity of peroxidase enzyme at 420 nm was determined by the method of Reddy *et al.* [38], in which oxidation of substrate catechol was done with the presence of H_2O_2. The change in absorbance measured in µmoles/min/mg protein.

Glutathione peroxidase

The glutathione peroxidase enzyme is used to decompose H_2O_2 or other peroxides as well as oxidize GSH in to GSSG. The activity of this enzyme was measured at 412 nm using the method of Rotruck *et al.* [39].

Glutathione reductase (GR)

Glutathione activity was measured at 340 nm using the method of David and Richard [40], in which glutathione was reduced to generate reduced glutathione (GSH) in the presence of NADPH, and NADPH was oxidized to form NADP+. One unit of glutathione reductase activity was defined as the reduction of 1 micromole of glutathione per minute per mg of protein (µM NADPH oxidized /minute/mg protein).

Glutathione S-transferase

According to the method of Habig *et al.* [41], 2, 4-dinitro-chlorobenzene (CDNB) react with reduced glutathione (GSH), to turn out a yellow product with 340-360 nm absorbance. Activity of enzyme related with the formation of product and it can be calculated by the increase in absorbance at 340 nm. The unit expressed in µmoles of 2, 4-dinitrophenyl glutathione formed /min/mg protein.

mRNA expression studies

Silk gland tissues were dissected out using an ice-cold PBS buffer. TRIZOL (Invtrogen) reagent method was used for the isolation of total RNA from desired tissues and quantity measured by using Qubit™ 4 fluorometer. The 1-5 µl of total RNA was used as template for first strand cDNA synthesis using iScript cDNA synthesis kit (Biorad) according to the manufacturers' instructions.

Table 1 Primers for real time PCR analysis

Genes	Primer (5'-3')	Reference
BmApaf-1	F: GGTTTGCTCGTAATGGAC	[43]
	R: CAGGACCAGTGGAGGCT	
BmDredd	F: AGTGACAGAAATGCTTGGAAC	[43]
	R: AAATGGGAACCTGAGGATG	
BmActin	F: CGGGAAATCGTTCGTGAT	[31]
	R: ACGAGGGTTGGAAGAGGG	

Real time PCR

RT-PCR was used to examine gene expression levels in this investigation. (Table 1) lists all primer sequences utilized in this study. The Biorad CFX Connect RT PCR apparatus and i Taq Universal SYBR Green Supermix were used to prepare RT-PCR experiments according to the manufacturer's instructions. 2µl of cDNA, 10µl of SYBR Green Supermix, 1µl of each gene specific primer and 4µl of ddH2O were used in each 20 µl reaction volume. The thermal cycler programme featured 5 minutes at 95 °C, followed by 10 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C and 10 s at 55 °C for 35 cycles. Every measurement was repeated three times. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ technique, which was described by Livak *et al.* [42]. *BmActin* was used as internal control.

Statistical analysis

The differences among control group and treated group were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using Origin statistical software and Microsoft Excel, the result obtained indicated as mean ± SEM (Standard error of mean). P values less than 0.05 considered as statistically significant.

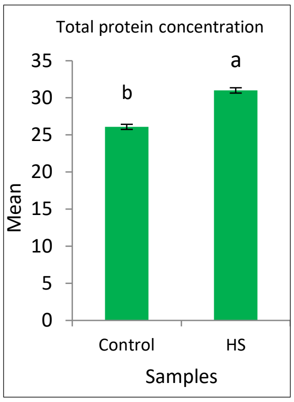

Fig 1 Effect of temperature on the protein concentration (mg/ml) of SG tissue. Those bars having different letters indicates significant difference (P < 0.05). The results represented as the mean ± SEM

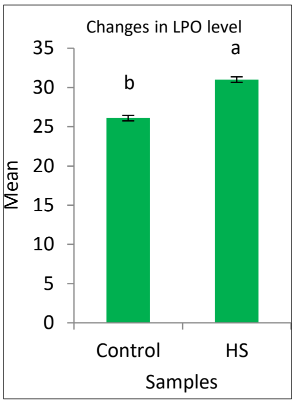

Fig 2 Effect of temperature on the level of MDA (µmoles/ min/ mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

RESULTS AND DISCUSSION

1. Total protein concentration of silk gland

Analysis of total soluble protein concentration in the silk gland showed significant increase in treated (2.16) group compared to the control (1.89).

2. Changes in LPO levels

MDA concentration indicates the level of lipid peroxidation in response to thermal stress, and it also describes the level of oxidative stress in the silk gland tissues of *B. mori* as shown in (Fig 2). The present study showed that there is a significant increase in the level of LPO in the treatment group compared to control.

3. Activities of antioxidant enzymes

The activity of SOD showed a significant increase in the test group compared to the control (Fig 3). The activity of catalase in the silk gland tissues of the control and test group was analyzed and found significant increase in the heat-exposed group compared to the control (Fig 4). The activity of POD enzyme in control and treated larvae of silk gland shows a very low level of activity in the control group but a significantly increased activity was found in the treated sample (Fig 5). GPX activity was significantly enhanced in the silk gland of *B. mori* larvae by exposure to temperature. Test group shows increased activity of GPX than the normal group (Fig 6). Analysis of GST activity showed discrepancy among the both examined groups, significantly elevated activity was found in the treated group than the normal group (Fig 7). Glutathione reductase shows lowest activity in the normal group but significantly increased activity was found in the treated sample (Fig 8).

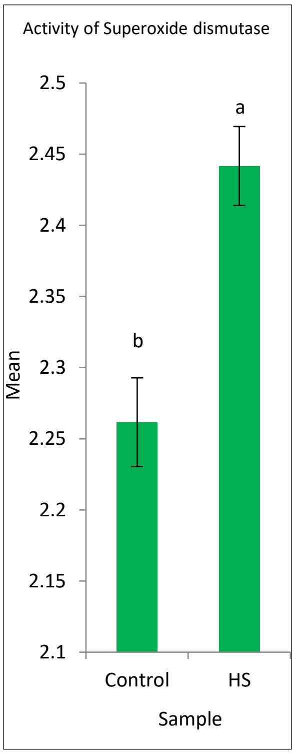

Fig 3 Effect of temperature on the activity of SOD (µmoles oxidized/min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

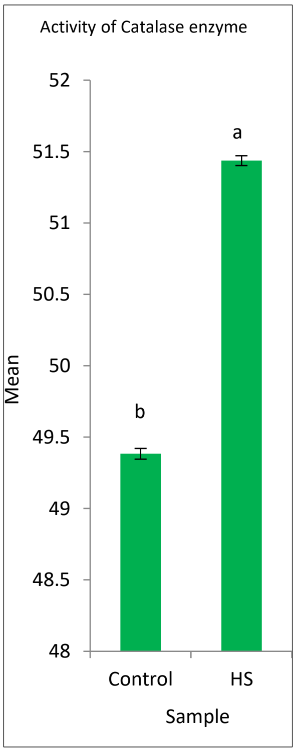

Fig 4 Effect of temperature on the activity of CAT (µmoles of hydrogen peroxide consumed/min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

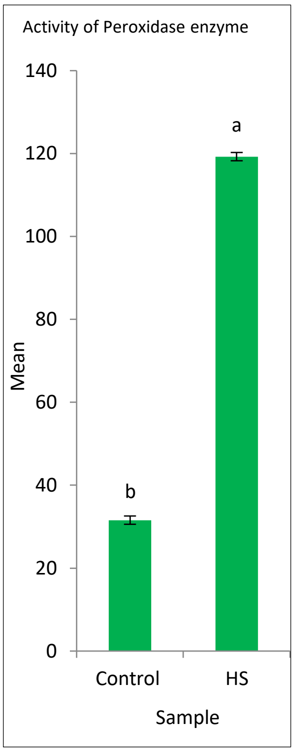

Fig 5 Effect of temperature on the activity of POD (µmoles /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

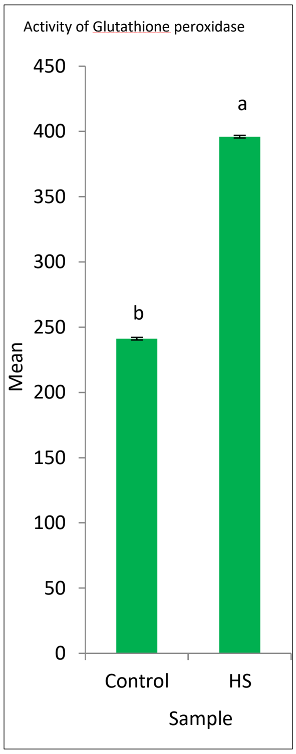

Fig 6 Effect of temperature on the activity of GPX (µmoles NADPH oxidized /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different. Data expressed as mean ± SEM

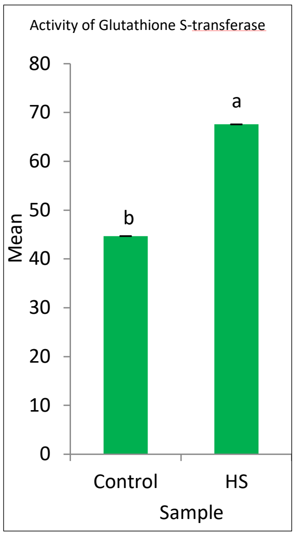

Fig 7 Effect of temperature on the activity of GST (μmoles /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different.
Data expressed as mean ± SEM

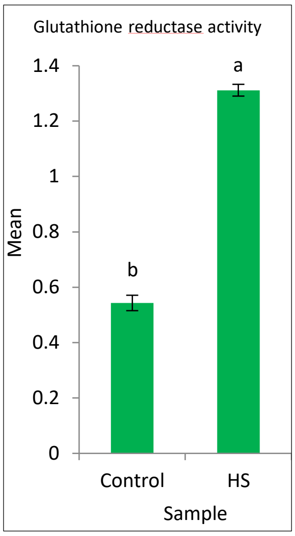

Fig 8 Effect of temperature on the activity of GR (μmoles of NADPH oxidized /min/mg protein) in SG tissue of *B. mori.* The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different.
Data expressed as mean ± SEM

Effect of thermal stress on the expression of BmApaf-1 and BmDredd genes

To evaluate the expression of mRNA levels of BmApaf-1 and BmDredd genes in silk gland tissues of the fifth instar larval stage by RT-PCR. The silk gland tissue showed change in the expression of BmApaf-1 and BmDredd genes in both control and test groups. The larvae exposed to thermal stress showed 1.7 fold significant upregulation of BmApaf-1 and 1.5 fold significant upregulation of BmDredd gene compared with control (Fig 9).

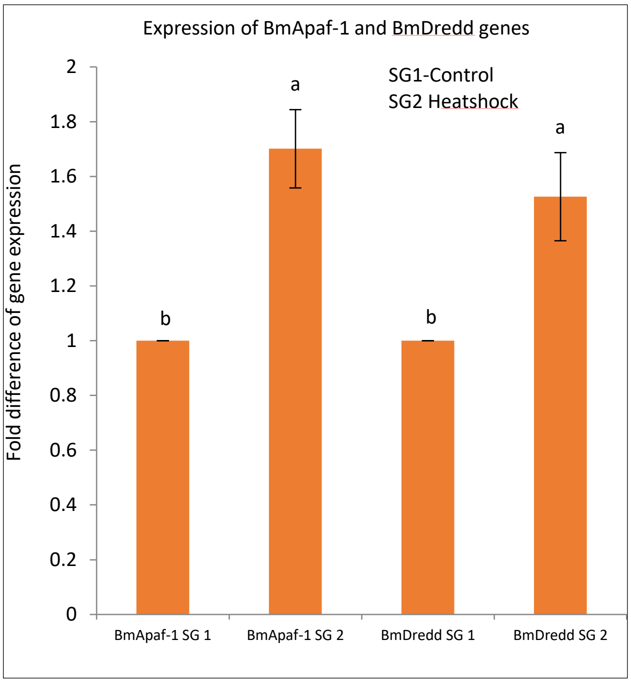

Fig 9 Effect of temperature on the expression of apoptotic related genes in SG tissue of *B. mori.* BmActin were used as the internal control to normalize the data that were represented as mean ±SEM by the three independent measurements. The 2^-ΔΔCT method was used to calculate the fold difference of gene expression. The bars sharing same alphabets are not significant. P < 0.05 represented as significantly different

Temperature is one of the major abiotic factors that influences the growth and development of silkworm and production of excellent quality cocoons [44]. Rates of biological as well as physiological processes have been adversely affected by high temperature [45], which will be reflected in the quality of cocoons [46]. The present study was intended to investigate the quantity of protein, level of oxidative stress, response of antioxidant enzymes and expression of apoptotic related genes against induction of thermal stress in the silk gland of silkworm. The quantity of protein in the larvae showed a significant increase in the silk gland in response to high temperature compared to the control. The results indicated that the larvae treated with high temperature exhibited elevated metabolic rate of proteins in the silk gland and may be due to the synthesis of new stress proteins. A similar inclination was demonstrated in tasar silkworm *Antheraea mylitta* D [47].

An exact steadiness exists between ROS production and activity of antioxidant enzymes, nevertheless this will be disturbed by environmental stress like high temperature disclosure, insect's exhibit consequent physiological responses lead to the generation of surplus ROS, which is able to smash up the structure of proteins, lipids, DNA and RNA [7] and also disrupt the cell membrane fluidity and in turn apoptosis [48].

Cellular and tissue level oxidative stress has been established by measuring the products formed by lipid peroxidation in vertebrates as well as in invertebrates. The products of lipid peroxidation is a sign of oxidative damage to lipids and in addition, it also triggers the regulation of antioxidant resistance mechanisms' [49]. An oxidative attack on the peroxidized polyunsaturated fatty acids gives the product MDA, an indicator of the extent of oxidative damage of lipids [50]. An elevated level of MDA concentration in the silk gland of fifth instar larvae was observed in response to thermal stress (Fig 2). Our results obviously demonstrated that in *B. mori*, thermal stress was accompanied by lipid peroxidation and other responses to oxidative stress. A Similar trend of increase in the levels of MDA was reported in the posterior silk gland, midgut and fat body of silkworm *B. mori* on exposure to azaserine [51]. More over the same trend was also reported in some other insects such as citrus red mite *Panonychus citri* [52], oriental fruit fly *Bactrocera dorsalis* [3], and predatory mite Neoseiulus cucumeris [53].

Antioxidant enzymes work synergistically to alleviate the oxidative stress engendered by surplus ROS inside the cell [53]. SOD, CAT, GST, GSH-Px, are enzymatic antioxidants and GSH is the non-enzymatic antioxidant. Among these antioxidants SOD is the most prominent ubiquitous metalloenzyme facilitating the diminution of elevated levels of superoxide radical induced by low or high temperatures [54]. The current study has revealed an increase in SOD levels in silk glands of fifth instar larvae exposed to temperature compared with control. This implies that the level of SOD was altered with changing environmental temperature. A parallel trend of result was demonstrated by Jena *et al*. [49], who observed that, a significantly elevated level of SOD in pupal testes of tropical tasar silkworm *Antheraea mylitta* under thermal stress. Ali *et al*. [55] reported that another insect *Mythimna separata* induced with high temperature showed significantly increased levels of SOD.

CAT is responsible for the degradation of H_2O_2 [49]. In the present study CAT activity was increased up on thermal stress in the fifth instar larvae (Fig 4). The result indicated that the relation between H_2O_2 production and CAT activity was influenced with thermal stress. Such a case was reported by [56], the CAT level in *Chilo suppressalis* larvae exposed to thermal stress significantly elevated over control. The result is also consistent with the findings of Jena *et al*. [49], they revealed that the activity of CAT is enhanced in the testes of pupa on exposure to thermal stress.

Glutathione s-transferases are known to be an important multifunctional enzyme play major role in the removal of toxic LPO products [53], detoxification of xenobiotics, protection from attack of oxidative stress and also help in isomerization and intracellular transportation [57]. In the present experiment, enhancement of GST activity was determined under thermal stress compared to the control. This suggests that GST actively eliminated the lethal LPO products formed on thermal stress [58].

POX enzyme is able to convert H_2O_2 into water and molecular oxygen [14], although it works together with GSTs and metabolizes the lipid peroxides [3]. In the present study, peroxidase enzyme shows increased activity under thermal stress. Similar observations were done by [53], [58]. These observations indicate the necessity of POX activity for scavenging the ROS produced under thermal stress.

Glutathione peroxidase is able to reduce free radical damage by metabolizing lipid peroxidation products and H_2O_2 using reduced glutathione as substrate [59]. The present data obtained in the GPx activity in *B. mori* larvae treated with temperature determined a significant increase compared with control. This data suggests that GPx takes major action against ROS produced during thermal stress [60].

The homodimeric flavoprotein GR is capable of synthesizing reduced glutathione (GSH) from oxidized glutathione (GSSG) using NADPH as a reducing cofactor. This bears different functions including coping against oxidative stress and also the biosynthesis of protein and DNA by maintaining a balanced ratio of GSH and GSSG [61]. In our study GR showed an increased activity in thermal stress induced groups than control [62].

Under stress conditions cells are capable of producing ROS and they could be a reason for inducing apoptosis [56]. ROS is capable of stimulating intrinsic as well as extrinsic pathway of apoptosis [21]. In the present study there was an enhanced production of MDA in the treated group indicates the lipid membrane damage due to ROS produced during the exposure of thermal stress. The present study implies an elevated expression of BmApaf-1 and BmDredd in the thermal stress exposed group compared to control. BmApaf-1 is involved in the intrinsic pathway of apoptosis. Chen *et al*. [63] reported that H_2O_2 induced oxidative stress in BmN-SWU1 cells showed an increased expression of BmApaf-1. Previous studies proved that BmDredd is actively participated in the apoptosis process; such a case was reported by Wang *et al*. [64]. An elevated expression of BmDredd was shown in the emodin induced treatment of BmN –SWU1 cells. UV treated BmN cells also showed increased expression of BmDredd in silkworm *Bombyx mori* [65].

CONCLUSION

In conclusion, thermal stress is able to produce oxidative stress in the silk gland of *Bombyx mori* larvae and it may cause lipid membrane damage. The antioxidant enzymes make an effort to reduce the oxidative stress by increasing their activity. The significant 1.7 fold increase of BmApaf-1 and 1.5 fold increase of BmDredd genes in heat induced silk glands may be the indication of activation of apoptotic pathway. Further investigations are required to learn more about impact of thermal stress on the apoptotic pathways in the silk gland.

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