5. DISCUSSION

The results derived from the present investigation in respect of heat shock at different developmental stages of new bivoltine strains of *Bombyx mori* L. and its effect on biological, biochemical and commercial traits is discussed in comparison with other allied species.

Our approach in the present study is different from that of others work keeping in view the introduction of new bivoltine hybrids for large scale production of cocoons for reeling in place of *F*₁ hybrids (PM × NB₄D₂) in tropical zones of India. Hence, here, an attempt has been made to determine the level of thermotolerance, expression of heat shock proteins to acquire thermotolerance and their influence on biological and commercial traits of bivoltine breeds NB₄D₂, NP₂, KSO₁, CSR₂ and CSR₄.

In the present study the well acclimatized bivoltine strain NB₄D₂ was considered as positive control where in heat shock response and heat shock proteins have been analysed by Joy and Gopinathan (1995). The heat shock response and hsp₄ that were expressed in new bivoltine strains NP₂, KSO₁, CSR₂ and CSR₄ was analysed for the first time and discussed on the light of additional new findings of the present study on NB₄D₂ with that of observations made by Joy and Gopinathan (1995).
5.1. Impact of heat shock at larval stage

5.1.1. Biological traits: Mortality

Our studies have revealed that the heat shock response in five bivoltine breeds varies with different developmental stages. Of the five instars, it is interesting that the young age silkworms that includes I, II and III instars are relatively sensitive to any given heat shock temperature while late age silkworm tolerate high temperature for relatively longer periods as also opined by Joy and Gopinathan (1995). Notably, the thermotolerance increases as larval development proceeds as in the order of V > IV > III > II > I in all the silkworm strains. However the highest of 21 percent mortality was observed (it includes the data of 35 and 40°C heat shock) in first instar larvae of CSR$_4$ and cent percent survivability in fifth instar. Whereas NP$_2$ exhibited comparatively least mortality in I instar and cent percent survivability in V instar. This high thermotolerance in fifth instar appears to reflect its adaptation to high ambient temperature that may encounter in course of its normal life. The phenomena that derived from the present study raise serious queries over the present rearing packages that young age silkworms are recommended to rear under high temperature and high relative humidity (RH), whereas late age silkworms to rear under low temperature and low RH. In such case, at field in tropical zones, where the environmental fluctuation does occur from dawn to dusk, temperature rises above 40°C for two hour that induces sudden shock in the larval system, resulting in larval mortality. The probability of the fact is that why farmers loose their cocoon
crops during summer, is undoubtedly due to cell damage that is caused by elevated temperature for short duration of 1-2 hours.

Exposure of different instar larvae to varied temperature ranging from 35 to 45°C for 2 hours has profound effect on biological features of new bivoltine strains. However though 40°C induces more mortality in first instar than 35°C but it was not true with fourth and fifth instars. Increase in heat shock temperature to 45°C severely affected survivability (100% mortality) at all instars of all silkworm strains. Comparatively fifth instar larvae of *Manduca sexta* another lepidopteron exhibit cent percent survivability at 42°C but zero percent survivability reported as heat shock temperature increased to 48°C (Fittingoff and Riddiford, 1990). In case of Pure Mysore (a multivoltine strain of *B. mori*) zero percent mortality occur at 42°C (for 1 hour) and cent percent mortality at 46°C (Lohmann and Riddiford, 1992b). Further, Hsieh *et al.* (1995) observed 100 percent mortality at 46°C (1 hour) among few Chinese and Japanese silkworm strains. In comparison with other insects, the threshold temperature that induces 100 percent mortality is 45°C in NB4D2, NP2, KSO, CSR2 and CSR4 (present study), 46°C in Chinese, Japanese (Hsieh *et al.*, 1995) and Indian (Pure Mysore- Lohmann and Riddiford, 1992b) silkworm strains, 48°C in *Manduca sexta* (Fittingoff and Riddiford, 1990), 40°C in *Drosophila melanogaster* (Lindquist, 1986), 48°C in *Lucilia cuprina* (Tiwari *et al.*, 1995), 46°C in *Musca domestica* (Tiwari *et al.*, 1997) and 50°C in *Locusta migratoria* (Qin *et al.*, 2003). Thus it is obvious that the degree of thermotolerance varies among different strains that are derived from same race or among different races that are geographically distinct.
Further, it is well known that the multivoltine silkworm strains are tolerant to high temperature than bivoltine strains (Hsieh et al., 1995; Joy and Gopinathan, 1995). But a comparative analysis on heat shock response among multivoltine (Joy and Gopinathan, 1995) strains with respect to heat shock is mere and among bivoltine it is confined to few Chinese and Japanese strains (Hsieh et al., 1995). Comparatively, among exotic bivoltine strains, the Chinese race, Feng is the most tolerant strain followed by the Japanese races, Kuo and J-09. The Chinese race C-54 is the most susceptive one. Thus either Chinese or Japanese thermotolerant strains can be used to cross with NB₄D₂, NP₂ and CSR₂ for production of thermotolerant hybrids.

However, information on heat shock response of new bivoltine breeds (NP₂, KSO, CSR₂ and CSR₄) that are released to field in place of NB₄D₂ is not available. Thus the present study is unique in this regard. Among these bivoltine strains, although NB₄D₂ is well acclimatized and has been exploited for production of F1 hybrids since long time, NP₂ exhibit better tolerance than that of other new bivoltine strains. Alternatively between CSR₂ and CSR₄ that are evolved and released by CSR&TI, CSR₂ has high thermotolerance than CSR₄. Hence our observations very well coincides with that of recent views (Dandin et al., 2004) that in case of inadequate production and availability of bivoltine cocoon of NB₄D₂, the CSR₂ strain which combine well with Pure Mysore (PM) strain could be used as male parents for the production of cross breed layings. Keeping in view the impact of CSR₂ as male component and performance of Kolar gold (PM × CSR₂) as promising
commercial breed (CB) combination as experienced by the farmers, the above approaches would keep both bivoltine and CB programme in right perceptive to improve cocoon yield and silk production (Dandin et al., 2004). Similarly, it is highly suggestive that the entire silkworm germplasm resources shall be subjected for systematic evaluation not only to determine thermotolerance before any strain/breed is selected as better parents for synthesis/evolution of suitable silkworm strains for tropical zones of India but also to consider them as male component to cross with that of PM to achieve many cross breeds as that of Kolar gold (PM x CSR,) which has led to crop stability under varying climatic conditions besides the high cocoon yield and better silk recovery when compared to PM x NB4Dr.

5.1.2. Analysis of heat shock protein expression at larval stage

Action of heat shock at varied temperature on total protein profile of different instars of different strains of Bombyx mori were analysed.

In the present study, we have reported expression of several heat shock proteins, of which 90 kDa heat shock protein expressed was reported for the first time from I, II and III instars, 84 kDa hsp from IV instar and 84, 62, 60, 47 and 33 kDa hsps in V instar silkworm larvae of NB4D2, NP2, KSO, CSR, and CSR4. We have not noticed repression of any other proteins on heat shock at 35 and 40°C at any stage. However, inhibition of normal protein synthesis observed at 45°C. Even Abromova et al. (1991) was also reported repression of fibroin protein in silk gland on induction of heat shock. Expression of only one 90 kDa protein in young age (I, II and III instar) and 4-5 hsps in V instar appears interesting and need to be addressed to understand
the molecular mechanism underlying developmental stage-specific expression of hsp in *B. mori*. This differential expression of hsp at different instars correlates well with the survivability of silkworm larvae. The zero percent mortality observed in fifth instar in the present study might be influenced by expression of different hsps. Thus it can be speculated that all these hsps collectively facilitate the silkworm larvae to acquire thermotolerance against the heat shock. Although differential expression of hsp at different instars and its biological significance is unexplored in silkworm but it can be used as set protocol for induction of heat shock during evaluation of thermotolerant silkworm strain or to select as better parents for evolution of new silkworm strain for tropics. Thus heat shock associated breeding programme might help in evolution of better hybrids for commercial exploitation.

We report 90 kDa hsp from I, II and III instar larvae of all silkworm strains for the first time on 35 and 40°C heat shock with over expression of 70 kDa protein on 35°C heat shock. Additionally, 84 kDa protein alone expressed in IV instar larvae of all the silkworm strains. Interestingly, expression of several hsps in V instar varies among silkworm strains. We have observed 84, 62, 60, 47 and 33 kDa hsps in NB4D2, and KSO, 84, 47 and 33 kDa hsps in NP, 84, 60 and 47 kDa hsps in CSR2 and only 84 and 47 kDa hsps in CSR3 silkworm strains. However, 84, 70, 31, 30 and 29 kDa hsps were reported form Pure Mysore (42°C heat shock for 1 hour) (Lohmann and Riddiford, 1992b). 83, 80, 74, 70, 68, 25 and 23 kDa hsps were expressed in cells and organs of Pure Mysore strain (Evegen’ev et al., 1987). 93, 46 and 28
kDa from Pure Mysore (Multivoltine), 93, 70, 46 and 28 kDa from C. Nichi (Multivoltine), 93, 89, 46 and 28 kDa from NB₄D₂ (Bivoltine) (Joy and Gopinathan, 1995). Interestingly, expression of 62 and 60 kDa protein in V instar is a unique finding of the present study. With these observations it is necessary to screen all the silkworm germplasm to establish strain/breed specific expression of hsps.

The difference in the pattern of expression of hsps in different instars of different new bivoltine silkworm strains is obvious. The expression of the different hsps in different tissues also varies depending on the stage of development or even the temperature at which the exposure was given (Joy and Gopinathan, 1995). The total concentration of hsps and their redistribution to specific subcellular sites are considered as more important factors in the acquisition of thermotolerance (Kampinga, 1993). Integument consisting of the cuticle and the underlying epidermis are the external tissue that acts as initial defense organ in most insects. Response of the integument to heat shock will therefore be an integral part of the survival of the silkworm. Thus our approach considering the whole larval body at different instar gave an insight for acquired thermotolerance that varies in different silkworm strains.

5.1.3 Sex specific expression of hsps

It is interesting to note that of the hsps expressed at different instars, intensive synthesis of a protein with molecular weight of 84 kDa occurs in female V instar larvae, while in male it is almost absent. In addition, a new
set of 62, 60, 47 and 33 kDa hsps were also expressed in female fifth instar larvae subjected to heat shock at 40°C, whereas these hsps were unnoticed in male larvae of the same age. But the preservation and intensification in expression of sex specific heat shock proteins varies among the silkworm strains studied. The female larvae of NB₄D₂ exhibited all these hsps than other strains. In order to test these differences associated with the sex of caterpillar, we sorted out V instar larvae based on sex markings, as it is difficult to differentiate sex phenotypically at earlier stages. Interestingly, the results obtained in the present study agree with the preliminary data concerning sex differences in the spectrum of B. mori proteins in different tissues and organs (Evgen’ev et al., 1987). This sex specific expression of hsps has not been reported earlier in any one of the organisms probably due to limitation in sex identification and differentiation. In case of B. mori, sex of the larvae could be easily differentiated based on sex markings seen after fourth moult and it is possible to subject male and female larvae separately for heat shock and sex specific expression of hsps can be determined with ease.

We propose here based on our observations that the expression of hsps in B. mori is maternally inherited as evident from the fact that resistance to high temperature associated with the female parent, whereas commercial characters of the cocoons are associated with the male parent. Hence, while production of FI hybrids normally multivoltines known for thermotolerance, be used as female parent to cross with the strain known for commercial traits as male parent which is susceptible to high temperature. (Suresh Kumar et al., 2001).
Thus this approach and protocol has unique feature and it can be successfully employed for selection of better parents while evolving thermotolerant silkworm strains through conventional breeding programme.

5.1.4 Glycoprotein and hsps

We established here for the first time that the hsp 84 and 33 are glycoprotein in nature in B. mori. As is seen from the figure 10, the hsp with molecular mass of 84 and 33 are distinctly stress glycoproteins but the functional significance remains to be defined. However, cellular stress conditions are known to elevate hsp synthesis and protein glycosylation leading to the development of cellular thermostolerance. The consistent association of stress glycoproteins with nonglycosylated hsps suggests a structural/functional role for protein chaperone complexes that consist of denatured proteins and the glycone/aglycone elements of cellular stress response (Jethmalani and Henle, 1998). Unfortunately no studies have yet demonstrated a link between glycoprotein and allozyme variation and thermal tolerance in B. mori.

5.1.5 Heat shock response on commercial traits of the cocoon

Interestingly, to date investigations have focused primarily on the HSP70 family and most hsps function as molecular chaperones that help organisms to cope with stress of both an internal and external nature. Recently the relationship between hsps and life history traits is explored with special emphasis on the ecological and evolutionary relevance of hsps (Sorensen et al., 2003). However, the relationship between hsps and commercial traits has not been explored in any of the organism studied so
far as they have got limitation from the point of commercial application. So in order to bridge the gap, the silkworm *B. mori* provides us a lot of scope to study the relationship between heat shock – heat shock proteins – stress resistance – adaptation to the environment – commercial traits. Thus the relationship between heat shock – heat shock protein – cocoon traits of *B. mori* is discussed. Notably, 17.52 and 13.48 percent of cocoon weight increased in NB$_4$D$_2$ over its control at 35 and 40°C respectively. Correspondingly, there was 19.44 and 13.45 percent improvement in shell weight at 35 and 40°C heat shock. Concurrently, the other counterpart CSR$_2$ also exhibited 13.11 and 6.44 percent of improvement in cocoon weight (Table 5b) and 16.26 and 5.03 percent improvement in shell weight (Table 6b) at 35 and 40°C over their respective controls. This increased cocoon and shell weight in NB$_4$D$_2$ and CSR$_2$ can be undoubtedly attributed that of heat shock and expression of heat shock proteins expressed at larval stage play a vital role in traits improvement. Further representatives of hsp family that are expressed in all the instars and in abundance particularly in fifth instar in NB$_4$D$_2$ and CSR$_2$ could induce tolerance to environmental insults during rearing as a consequence the heat shocked larvae can overcome the environmental insults and could spin better cocoon than their respective controls. Though Joy and Gopinathan (1995) reported that there is no effect of heat shock on the commercial traits but after heat shock if these larvae are reared under normal environmental conditions where frequent fluctuation does occur, the heat shocked larvae can overcome the subsequent environmental stress and spin good quality cocoon than the non heat shocked populations.
In the present study, the increased cocoon weight and shell weight over control reflects the positive correlation between heat shock and expression of hsp with that of silk protein content in the cocoon. But repression of synthesis of fibroin in the silk gland on heat shock (Abramova et al., 1991) offers further systematic investigation in different breeds of silkworm. However, no larvae were recovered from heat shock at 45°C at any instar to spin cocoons. Concomitantly, though cocoon weight and shell weight in NP, KSO, and CSR was not significantly increased as that of NB, D and CSR but the shell ratio found to be better in NP at 35°C and in CSR at 40°C. The differences between cocoon weight and shell weight and intum shell ratio over different silkworm strains after heat shock need further investigation to determine the factors responsible for it.

5.2 Determination of sensitivity to heat shock at pupal stage

5.2.1 Impact of heat shock on mortality

The pupa of NB, D, and new bivoltine hybrids of B. mori was found to be more tolerant compared to larvae of the same strain as has been reported in Pure Mysore, C. Nichi and NB, D (Joy and Gopinathan, 1995) and in Lucilia cupriana and Musca domestica (Tiwari et al., 1995 and 1997).

In the present study an average of 0.0, 7.0 and 38 percent mortality was recorded at 35, 40 and 45°C heat shock at pupal stage compared to larval stage where cent percent mortality noticed at 45°C. Better thermotolerance in pupa than larvae may be because of thick exoskeleton covering and significant low physiological and metabolic status.
5.2.2 Expression of heat shock proteins

Though pupae showed good resistance even at high temperature than larvae, they did not express heat shock protein. But, Joy and Gopinathan (1995) observed expression of 93 kDa protein from pupa of *B. mori* heat shock at 41°C for 1 hour. Contrary to this in the present investigation neither expression nor over expression of heat shock protein was seen in any of the silkworm strains. The molecular phenomena for not expressing the hsp need to be studied.

5.2.3 Impact of heat shock on fecundity and hatching percentage

There was no much difference in the fecundity of NB$_4$D$_2$ and KSO$_1$ strains in response to heat shock compared to their controls, probably as pupa exhibit high resistance to heat shock. Interestingly, NP$_2$ recorded 7.52 and 13.69 percent improvement at 35 and 40°C heat shock followed by CSR$_3$ (7.11 and 7.78 %) and CSR$_4$ (3.31 and 5.25 %) over their respective controls (Table 8). This clearly indicates that as NP$_2$ is found to be sensitive to heat shock at larval stage, similarly the pupa influenced by heat shock and responded positively. This varied response at larval and pupal stage of a strain in respect of thermotolerance and thermosensitive could be explored properly. Further as the pupal stage found to be resistant to heat shock up to 45°C the process of oogenesis is not affected as a result hatching percentage remain normal.