TEMPERATURE INDUCED CHANGES IN THE BIOLOGY AND HEAT SHOCK PROTEIN GENE EXPRESSION IN MALATHION RESISTANT RED FLOUR BEETLE, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

by

SANJAY SABU
(2019-11-230)

DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

2023

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THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

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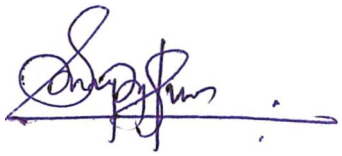
DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE,
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

2023

DECLARATION

I hereby declare that the thesis entitled "**Temperature induced changes in the biology and heat shock protein gene expression in malathion resistant red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)**" is a *bona fide* record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.



Place: Vellanikkara
Date: 24. 02. 2023

Sanjay Sabu
(2019-11-230)

CERTIFICATE

Certified that the thesis entitled **"Temperature induced changes in the biology and heat shock protein gene expression in malathion resistant red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)"** is a *bona fide* record of research work done independently by **Mr. Sanjay Sabu (2019-11-230)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship, or associateship to him.

Place: Vellanikkara
Date: 24. 02. 2023

24/02/23.

Dr. Mani Chellappan

(Chairman, Advisory Committee)
Professor and Head
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Sanjay Sabu (2019-11-230),** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology**, agree that this thesis entitled **"Temperature induced changes in the biology and heat shock protein gene expression in malathion resistant red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)"** may be submitted by **Mr. Sanjay Sabu** in partial fulfilment of the requirement for the degree.

24/02/23.

Dr. Mani Chellappan

(Chairman, Advisory Committee)
Professor and Head
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara.

24/02/2023

Dr. Abida P.S

(Member, Advisory Committee)
Professor and Head,
Department of Plant Biotechnology, CPBMB
College of Agriculture, KAU
Vellanikkara, Thrissur

24/2/2023

Dr. Berin Pathrose

(Member, Advisory Committee)
Associate Professor
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara.

Dr. Deepu Mathew

(Member, Advisory Committee)
Professor
Department of Plant Biotechnology, CPBMB
College of Agriculture, KAU
Vellanikkara, Thrissur.

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SANJAY SABU

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ABBREVIATIONS

%	Percentage
ºC	Degree celsius
bp	Base pair
cDNA	Complementary DNA
Ct	Cycle threshold
DEPC	Diethyl pyrocarbonate
dNTPs	Deoxyribonucleoside triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
FCI	Food Corporation of India
g	Gram
h	Hour
Kb	Kilo base pairs
kDa	Kilo Dalton
kJ	Kilo joules
RNA	Ribonucleic acid

INTRODUCTION

1. INTRODUCTION

The human population has reached 7.9 billion and is growing at a rate of around 1.05 percent every year, with the current average population increase estimated at 81 million people per year. As the population tends to increase at an alarming rate, there's an urgent need to meet all the requirements, with food resources being the most important among them. With the total area under agriculture decreasing due to various climatic and anthropogenic interventions, the need of the hour has been to reduce post-harvest losses.

Post-harvest losses in cereals and pulses account for about 19 %, 20 % and 44 % of weight losses in cereal crops, root crops and fruit and vegetables respectively, and up to 53 % loss in calorific content in cereals (Gustavsson *et al.,* 2011). Among all the post-harvest damages caused to the stored products, insect pest damage accounts for the maximum loss. Red flour beetles (RFB), *Tribolium castaneum* (Herbst), is one of the most notorious storage pests and needs to be controlled at the storage facilities to reduce the losses. Both the adult and larval stages of the insect can cause damage to the stored products. They feed on cereals, flour, fruit nuts, starchy material, millets, prepared cereal foods and broken grains which results in dust formation. The infected product also emits a sour and pungent smell, due to the secretion of benzoquinones by the beetles, which makes the food article undesirable. Although there exist several pest management methods, application of insecticides remains the most widely used method to check the insect population since they are quicker, more effective, and economically sound to control the pest at an acceptable level. (White and Leesch, 1995; Perez-Mendoza,1999). The problem arises when there is an indiscriminate usage of insecticides. The traces or residues of insecticides in food articles can affect the food quality and may hinder the trade of the product too. Moreover, insects tend to develop resistance towards insecticides, which in turn results in relying on a higher concentration of insecticides to keep the infestation at a check.

In India, the insecticides recommended for management of storage pests in Food Corporation of India (FCI) godowns were malathion, dichlorvos and deltamethrin. Bhatia *et al.* (1971) reported the development of malathion resistance in red flour beetle accounting for around 37.76-fold in India. Since then, there have been different studies reporting the development of resistance towards malathion in different parts of

the country. A study on the status of malathion resistance in the beetle populations in different FCI godowns in Kerala showed that the strains collected from Angamaly, showed maximum resistance with an LC_{50} of value 6949.80 ppm. It was stated that the resistance may be attributed to the same protocol being followed by the FCI that frequently exposed these beetles to the same level of pesticide concentration and application frequency (Anusree *et al.*, 2019). All organisms including insects survive adverse conditions or stresses by altering different physiological functions. The limited available resources force the insects to take regulatory decisions to allot these resources from growth or reproductive pathways to defence pathways during the stress period. As a result, there is a decline in the synthesis rate of most proteins which can affect the normal growth and development of the insect. While certain proteins like heat shock protein (Hsp) expression increases, as in the case *Chortoicetes terminifera* where taxa-specific hsps were over-expressed when subjected to crowding (Chapuis *et al*. 2011). Hsps often protect the species from adverse conditions, however over-expression of hsps may also demand considerable fitness cost.

Zhang *et al.* (2015) showed that the insecticide resistance of *Xylostella plutella* was affected when exposed to heat treatment. The resistant strains were found to be susceptible to insecticides when exposed to extreme temperatures, which were attributed to the fitness cost due to over production of different HSPs and detoxifying enzymes. Therefore, the resistance towards agrochemicals developed was often achieved at a cost usually referred to as the fitness cost and has been documented in different species. (Bourguet *et al*. 2004; Berticat *et al*. 2008). Exploiting the fitness cost associated with insecticide resistance can help in controlling the pest population and to apply lower concentrations of the pesticide to manage the infestation. This can also save the produces from pesticide residue contamination. Hence this work is proposed with the following objectives:

1. Evaluation of susceptibility of malathion-resistant and susceptible strains of RFB to temperature stress.
2. Gene expression analysis of *Hsp70* in different stages of resistant and susceptible strains subjected to heat stress.
3. To study, if any trade off exists between temperature and chemical resistance in RFB

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The red flour beetle, *Tribolium castaneum* (Herbst) is a member belonging to the largest insect order, coleoptera, and is a menace when it comes to post-harvest products and storage facilities. They are cosmopolitan in distribution with Indo-Australian origin. These are polyphagous insects with short life cycles and high fecundity. The entire genome sequencing has been completed for this species (*Tribolium* Genome Sequencing Consortium, 2008), which has enabled the use of this species as a "genetic model" in many recent studies including RNA interference (RNAi), creation and exploitation of generic maps and immunological studies (Tomoyasu & Denell, 2004; Lorenzen *et al.*, 2005; Knorr *et al.*, 2009).

The literatures that were found relevant to this study were collected and arranged under the following topics:

2.1 Biology of the insect
2.2 Damage caused by the insect
2.3 Management of the insect
2.4 Resistance status in RFB
2.5 Mechanism of insecticide resistance
2.6 Fitness cost and trade-off
2.7 Temperature stress and heat shock proteins

2.1. Biology of the insect

Tribolium castaneum belongs to Tenebrionidae also called the "darkling beetles" group, having characteristic darker body color ranging from black to reddish brown (Singh and Prakash, 2015). The word "Tenebrio" literally means seekers of dark places (Haines, 1991). Richards *et al.*, (2008) sequenced the whole genome of the insect, which enabled this insect to be also used as a model insect for molecular studies and food safety studies (Grünwald *et al*., 2013).

Adult insects usually are flat and oval in shape measuring approximately 2.3-5 mm in size (Rees, 2004; Hagstrum,2013). They have a flattened body with parallel sides and a shiny reddish-brown exoskeleton with a smooth texture. These insects are sexually dimorphic, and the males can be distinguished from females due to the presence of "sex patches" (setiferous endocrine glands) present on the first pair of legs that aid in attracting females and in providing cues for aggregation in storage areas. (Hilton, 1942; Sokoloff,

1972). These insects have a pair of antennae composed of eleven segments with the last three segments enlarged or clubbed together (Baldwin and Fasulo, 2014), they are also equipped with functional hind wings that are used for flight (Ridley *et al.*, 2011).

The adults may have a life span of 3 years or more and follow a typical holometabolous life cycle. Under optimum conditions, with temperatures ranging between 20℃ to 37.5℃ and at R.H. greater than 70%, the development of adults from eggs took only 28 days (Howe, 1962). The incubation period varies from 4-12 days in accordance with the external conditions (Gentry *et al.*, 1991).

Sattigi *et al.*, (1995) after rearing the insects in rice, maize, sorghum, and wheat flour suggested that wheat flour was the most suitable feed and rice flour the least suited. In laboratory conditions, they usually live around 7-11 months with the fecundity of females declining due to the exhaustion of the germ cells after 3-4 months.

Mahroof *et al.* (2003) reported that there was no impact due to relative humidity on insect mortality of stages exposed to elevated temperature. The females reared in laboratory conditions tend to exhibit a higher reproductive potential compared to those found in the wild (Daglish, 2005). The maximum egg-laying capacity may be achieved from five to ten days even though these insects can achieve successful mating as early as 3 hours from emergence.

The female beetles lay around 200 to 500 eggs during their lifecycle. The eggs are microscopic, white in color and often are found to be sticking onto the food particles and walls of container and storage houses. The egg period may last around one week, 4-7 days (Devi and Devi, 2015) or about 2.7 days on an average (Haines, 1991). Normally, the eggs may measure about 0.61x 0.3mm (Leelaja *et al.*, 2007).

The eggs then hatch into slender, mobile larvae which are yellowish to brown in color and often seen within the damaged grains or flours (Ebeling, 2002). When fully grown the larva can be about 4-5mm long. They are campodeiform larvae with six to seven instar stages, with each instar having a characteristic feature to distinguish them from one another. They feed actively and are often dark dwellers, hiding in darkness or in cracks of products (Rees, 1996). The larval period and length of each instar vary depending on the type, quality and availability of food, ambient temperature, and relative humidity. Haines (1991) reported that the average length of the larval period was 12.9

days on average, whereas some studies showed the extending to 12-13 days (Howe, 1956) and 70-80 days (Devi and Devi, 2015).

The pupae of *T. castaneum* are white in color, immobile and unprotected. They do not feed on any material and therefore cause very little damage to stored products (Weston and Rattlingourd, 2000). They are yellowish in color but turn brown during the later stages, they are dorsally covered with fine hairs and have well-developed eyes (Beeman *et al.*, 2012). The pupae measure around 3.3-3.4 mm in length, 1.1 mm wide and weighs nearly about 2.4-2.5 mg. The characteristic shape of the venter of the last abdominal segment can be used to distinguish the sexes of the insect pupa (Sinha and Watters,1985). The pupal period extends to 6-7 days on average.

2.2 Damage caused by the insect

As few as ten species of the dozen species belonging to the order Tenebrionidae are storage pest associated grains which often belong to the groups, *castaneum, confusum* and *brevicornis*. They are incapable of causing damage to the whole grains, thus being the secondary pests for several cereals and pulses. These insects are commonly referred to as "flour beetles" or "bran beetles" because of the typical habitat they prefer, i.e., the stores of these grain flours (Nakakita, 1983).

Both larval and adult stages of the insect cause damage, and the product once attacked will accumulate exuviae and carcasses. The produce may also turn greyish upon infestation (Lale, 2002). The beetles secrete benzoquinones as part of a defense mechanism from their abdominal glands with an increase in their age, which can cause a strong pungent smell that can affect the quality and trade of the stored product. Severe infestation due to these beetles can also induce certain changes in the storage environment that prove to be conducive for certain fungi thereby aggravating the losses further (Sallam, 2008).

RFB is one of the most important pests of cocoa beans, both in farms and warehouses (Dharmaputra, 1999; Hamid and Lopez, 2000; Finkelman *et al.*, 2003; Navarro, 2012; Bateman, 2015). This polyphagous insect has been constantly reported in wheat and wheat flour products. They also attack cereal - based products like pasta, cornflakes, biscuits, nuts, beans, and even dry fruits (Hussain *et al.*, 1996; Kheradpir, 2014). They have also been reported to be attacking groundnuts/peanuts (Kheradpir, 2014), soyabean meals (Cox and Simms, 1978), yam, cassava and even damages reported

in dried specimens in museums (Dharmaputra, 1999; Ajayi and Rahman, 2006; Tettey *et al.*, 2014). Although these insects are said to cause certain allergic reactions, there aren't reports of any diseases caused in humans (Alanko *et al.*, 2000).

2.3. Management of the insect

Since RFB doesn't infest sound or undamaged grains and the degree of progeny development and survival of the beetles are reported to be higher in broken grains than in the whole grains (Lale and Modu, 2003). Therefore, the first step to reduce the infestation is to find and remove all the possible sources of infestation. Even the tiniest bits of broken grains can be a source for these beetles to feed and survive. The presence of leaky packages in the storage spaces, where small bits and pieces of grains and meals are spilled is often a sign of a possible infestation. All the infested materials need to be collected and disposed of after packing them into a sealed container. Since these beetles can survive in different temperature regimes, storing the infested materials in a freezer for more than four days can also reduce their survival (Arvogast,2000; Koehler, 2003).

The most commonly adopted method for control of storage pests in storage facilities is the chemical method. Malathion is one of the insecticides recommended for use in storage godowns of the Food Corporation of India (FCI) along with dichlorvos and deltamethrin. Some of the pesticides used and their consumption in the FCI godowns across India are given in Table 1. Malathion is an organophosphate that is being used intensively for routine protection for a long time against storage pests. Malathion and deltamethrin are recommended for spraying on the jute sacks while dichlorvos for floors, roofs, and walls of the storage godowns.

Malathion belongs to the group of organophosphates, which targets and inhibits Acetyl cholinesterase (AChE). The inhibition of this esterase which are essential for neurotransmission can cause paralysis and ultimately death of the insect. Although malathion is no longer being used as an insecticide in FCI godowns, malathion resistance and their cross-resistance effect are being reported (Couso-Ferrer *et al*. 2011).

Table 1. Pesticides used in FCI godowns and their consumption from 2017-2018 to 2020-2021. (In M.T. Tech. Grade)

Pesticide	Group	2017-2018	2018-2019	2019-2020	2020-2021
Aluminum phosphide	R*	76.42	94.59	91.85	103.57
Deltamethrin	I**	10.73	45.49	49.67	24.59
Dichlorvos	I**	287.11	344.05	537.05	35.42
Malathion	I**	103.00	656.41	647.14	305.41

R*: Rodenticide; I**: Insecticide (Source: IndiaStat)

Increasing health and environmental issues arising due to the indiscriminate use of conventional pesticide usage, there had been an intensification of research for finding an effective but safer control method for the management of different insects (Maharoof *et al.*, 2005; Kim *et al.*, 2015). Use of temperature as a method to control the pest population is one of the alternatives used in food processing and storage godowns for controlling pest incidence (Roesli *et al.*, 2003). The insects are subjected to sub-lethal temperatures by virtue of which their population can be controlled.

Islam and Talukder (2005) demonstrated that the use of neem seed extract and powdered marigold leaves along with commercial insecticides malathion and carbaryl could be a potential alternative insecticide against red flour beetles. While Britto *et al.* (2005) studied antifeedant properties of *Acorus calamus, Azadiracta indica, Ocimum sanctum, Punica granum* and *Zingiber officinale* on red flour beetle adults and suggested the tested plants could serve as an effective control agent and may be exploited for the same.

Conyers and Bell (2003) investigated the feasibility of modifying the atmosphere at storage facilities on five species that included *Tribolium castaneum, Cryptolestes ferrugineus, Sitophilus granarius, Oryzaephilus surinamensis* and *S. oqyzae*. This study reported that regulation of oxygen (O_2) and carbon dioxide ($CO_{2)}$ content in the facilities can help reduce the emergence of the insects i.e., increasing CO2 content by 10%-20% and reducing the O2 content to 5% was found sufficient to eliminate *S. granarius* emergence even at 20℃.

Kavallierratos *et al.* (2021) showed that the essential oil extracted from different parts of tancy, *Tanacetum vulgare* L. (Asteraceae) showed potential adulticidal and larvicidal effects on *T. castaneum, T. confusum, Tenebrio molitor* and *Oryzaephilus surinamensis*. The study showed that the essential oil showed higher mortality i.e., 100% and 94.4% mortality of *T. castaneum* larva at 1000 and 500ppm respectively. Using plant volatiles as an alternative to chemical pesticides has gained importance in recent years. Some of the essential oils extracted from different plant species that were reported to be effective against *T.castaneum* are given in Table 2.

Table 2. Different plant species and their extracts reported with potential activity against *T. castaneum*

Plant species	Formulation	Reference
Achillea wilhelmsii	Essential oil	Khani *et al.* (2012)
Acorus calamus	Essential oil	Talukder *et al.* (2009)
Agastache rugosa	Essential oil	Lee *et al.* (2020)
Allium sativum	Essential oil	Yang *et al.* (2010)
Amomum maximum	Essential oil	Wang *et al.* (2015)
Artemisia sp.	Essential oil	Liang *et al.* (2017); Bachrouch *et al.* (2015); Zhang *et al.* (2015)
Aster ageratoides	Essential oil	Chu *et al.* (2013)
Azadirachta indica	Essential oil	Adarkwah *et al.* (2010)
Carum sp.	Essential oil	Tawfeek *et al.* (2017); Ziaee *et al.* (2014)
Capsicum annuum	Plant extract	Lee *et al.* (2020)
Cayratia japonica	Essential oil	Liu *et al.* (2012)
Cinnamomum verum	Essential oil	Tawfeek *et al.* (2017)
Citrus medica	Essential oil	Luo *et al.* (2019)
Citrus reticulata	Essential oil Ethanol extract Powder	Iram *et al.* (2013); Lee *et al.* (2020)
Citrus sinensis	Essential oil	Saleem *et al.* (2013)

Calamintha glandulosa	Essential oil	Popoviæ *et al.* (2013)
Crithmum maritimum	Essential oil	Polatoğlu *et al.* (2016)
Cupressus lusitanica	Essential oil	Bett *et al.* (2017)
Cymbopogon sp.	Essential oil	Bossou *et al.* (2017); Tawfeek *et al.* (2017)
Dracocephalum moldavica	Essential oil	Chu *et al.* (2011)
Drimys winteri	Essential oil	Zapata *et al.* (2010)
Etlingera yunnanensis	Essential oil	Guo *et al.* (2015)
Foeniculum vulgare	Essential oil	Tawfeek *et al.* (2017)
Laurelia sempervirens	Essential oil	Zapata *et al.* (2010)
Mentha sp.	Essential oil	Khani *et al.* (2012); Salem *et al.* (2017); Pang *et al.* (2020)
Myristica fragrans	Essential oil	Tawfeek *et al.* (2017)
Ocimum sp.	Essential oil	Ogendo *et al.* (2008); Saleem *et al.* (2018)
Tagetes sp.	Essential oil	Krishna *et al.* (2005)
Thuja occidentalis	Essential oil	Abdelgaleil *et al.* (2016)
Zanthoxylum armatum	Essential oil	Zhang *et al.* (2017)
Zingiber purpureum	Essential oil	Wang *et al.* (2015)

Another novel technique that gained popularity in recent years is the genetic-based techniques of RNA interference (RNAi). This method can be more target-specific and lesser damaging to the environment (Baum *et al.*, 2007; Noh, Beeman and Arakane, 2012). Perkin and Oppert (2019) studied stage-specific expression of genes in *T. castaneum* and the identified cuticle protein gene (CPG) as a candidate gene that had the potential of being used as an insect control product using RNAi technology. They observed that the gene was expressed at lower levels in all insect stages except in the larval gut, which had a moderate level of expression, which suggested that oral delivery of CPG dsRNA can potentially control the *Tribolium* larval population, ultimately reducing the damage caused.

2.4. Resistance status in RFB

The red flour beetle has a reputation for developing resistance against nearly every pesticide used for its management. Srivastava *et al.* (2001) reported that out of thirteen populations of *T. castaneum* collected from National Seed Programme Centres across India, eleven strains were said to have developed resistance against malathion with the resistance level varying from 0.725 to 24.53 folds.

Rahman *et al.*, (2007) also reported that the samples of *T. castaneum* collected from one silo and eight different storage depots in Bangladesh showed resistance against all the chemicals used for its control namely, malathion, dichlorvos, phosphine, fenitrothion and pirimiphos-methyl. The study also indicated that the resistance against malathion was the highest among all other insecticides, accounting for nearly 18-fold resistance.

A study conducted in Serbia, evaluating the susceptibility of *T. castaneum* to various insecticides reported that malathion was the least toxic among the insecticides used in the study (Andric *et al.*, 2010).

Anusree *et al.*, (2019) studied malathion resistance in RFB across five different FCI godowns in Kerala, and reported that the population collected from Angamaly, Thrissur showed the maximum resistance to malathion with LC_{50} value 6949.80 ppm, which was followed by Mulangunnathkavu (6157.30ppm), Valiyathura (5873.02ppm), Olavakkode (5727.94ppm) and Thikkodi (5703.49 ppm).

2.5. Mechanism of insecticide resistance

Pesticide resistance is a dynamic and complicated process that is influenced directly by the insect pest's genetic, physiological, behavioral, and ecological characteristics. They are also influenced indirectly by operational factors such as insecticide types, application time, rate of application, coverage, and the procedure followed for the pesticide application (Carrière *et al.*, 2004; Onstad, 2008)

Insecticide resistance mechanism in *Aedes aegypti* has been reported to be achieved mainly by four mechanisms: (i) by increasing detoxifying enzymes activity (Hemingway *et al.*, 2004; David *et al.*, 2013), (ii) by knockdown resistance or target site insensitivity (Hemingway *et al.*, 2000; Soderlund *et al.*, 2003), (iii) behavioral avoidance

(Chareonviriyaphap *et al.*, 2013) and (iv) cuticle thickening (Wood *et al.*, 2010; Kasai *et al.*, 2014).

Knockdown resistance and metabolic resistance are considered to be the most important among the mechanisms that confer resistance to the insect. Metabolic resistance involves the over-expression or changes in the conformation of different enzymes which are essential for insecticide detoxification. Major classes of enzymes involved in insecticide detoxification are mixed function oxidases (MFO) which include enzymes like cytochrome P450 monooxygenases, glutathione-S-transferases (GST) and esterases/carboxylesterases (Shakoori *et al.*, 2000; Lui *et al.*,2015).

In *T. castaneum,* the P450-mediated response was found to be responsible for resistance against the insecticide, deltamethrin. Scientists have suggested that *CYP6BQ9* genes were involved predominantly in deltamethrin resistance (Zhu *et al.,* 2010). Further, Liang *et al.* (2015) reported eight more genes which also aided in deltamethrin resistance.

Jagadeesan *et al.* (2013) using genetic studies and next-generation sequencing reported that in *T. castaneum* there exist two unknown loci named *tc_rph1* and *tc_rph2*, that were associated with phosphine resistance, a fumigant used in storage sites for pest control. Polymorphism of an enzyme, dihydrolipoamide dehydrogenase (DLD) was also found evident in imparting phosphine resistance in these beetles (Schlipalius *et al.* 2012).

Pyrethroid and DDT Knockdown resistance is achieved by alteration in the genome due to single or multiple mutations in the genes that are encoding proteins mainly on those of voltage-gated sodium channels (VGSC). Pyrethroid resistance in virtue of *VGSC* gene modification is basically monogenic, while other metabolic resistance can be regulated by one or more genes (Prasad and Shetty, 2013; Dong *et al.,* 2014; Abbas *et al.,* 2014; Khan *et al.,* 2015).

Mutations arising due to point mutations, exon skipping, or truncated protein production were reported to be responsible for conferring spinosad resistance in *Drosophila*. The α6 subunit of the nicotinic acetylcholine receptor was found to be mutated however the mutation was found to be not “lethal” to the insect but rather conferred resistance against Spinosad (Baxter *et al.*, 2010; Rinkevich *et al.*, 2010; Hsu *et al.*, 2012; Puinean *et al.*, 2013; Bao *et al.*, 2014; Berger *et al.*, 2016)

2.6. Fitness cost and trade-off

Development of resistance is always beneficial for any organism, but this resistance usually is traded off with other important life traits. Insects usually develop resistance towards pesticides; however, the costs of developing resistance (known as fitness cost) are also documented in many species (Bourguet *et al.,*2004; Berticat *et al.*, 2008). *Plutella xylostella* (Sayyed and Wright, 2001; Reymond *et al.*, 2005; Liu *et al.*, 2008), *Aedes aegypti* (Martins *et al.*, 2012), *Anopheles gambiae* (Djogbenou *et al.*, 2010), *Myzus persicae* (Castaneda *et al.*, 2011; Silva *et al.*, 2012), *Culex pipiens quinquefasciatus* (Hardstone *et al.*, 2009) and *C. pipens* (Lonormandm *et al.*, 1999) are some of the insects in which considerable fitness cost has been reported.

In plants, growth is attributed to the growth of their vegetative tissues, which further determines the overall growth of the plant (Figure 1). Leaves and roots act as the source for important metabolites including carbohydrates and nitrogen and are then transferred accordingly to different sinks. The balance between the source and sink in plants is essential for their growth, however any stress (environmental factors, herbivory etc.) can hinder this balance forcing trade-off between important traits (White *et al.*, 2015). At least three non-mutually exclusive mechanisms could contribute towards trade-off between two life functions, which are (i) allocation cost, which arise when the costs are directly limited due to competition for resources, (ii) genetic costs, where pleiotropy or linkage equilibrium associated with the linked trait can be associated with trade-off and (iii) energetic limitations or ecological costs, where the plants environment can penalize co-expression of different traits (Coley, 1985).

In the case of insects, studies suggest that the depletion of resources is believed to be partially responsible for the fitness cost. Over-expression of detoxifying enzymes and other metabolites when subjected to any stress has also been reported to restrict available energy for other vital life functions (Higginson et al, 2005; Konopka *et al.*, 2012).

Foster *et al.* (2003) reported that in highly resistant clones of *Myzus persicae*, over-expression of the enzyme esterase-4 (E4) which aids in sequestering and hydrolysis of a wide range of insecticides showed a reduction in capacity to overwinter compared to moderately resistant and susceptible clones.

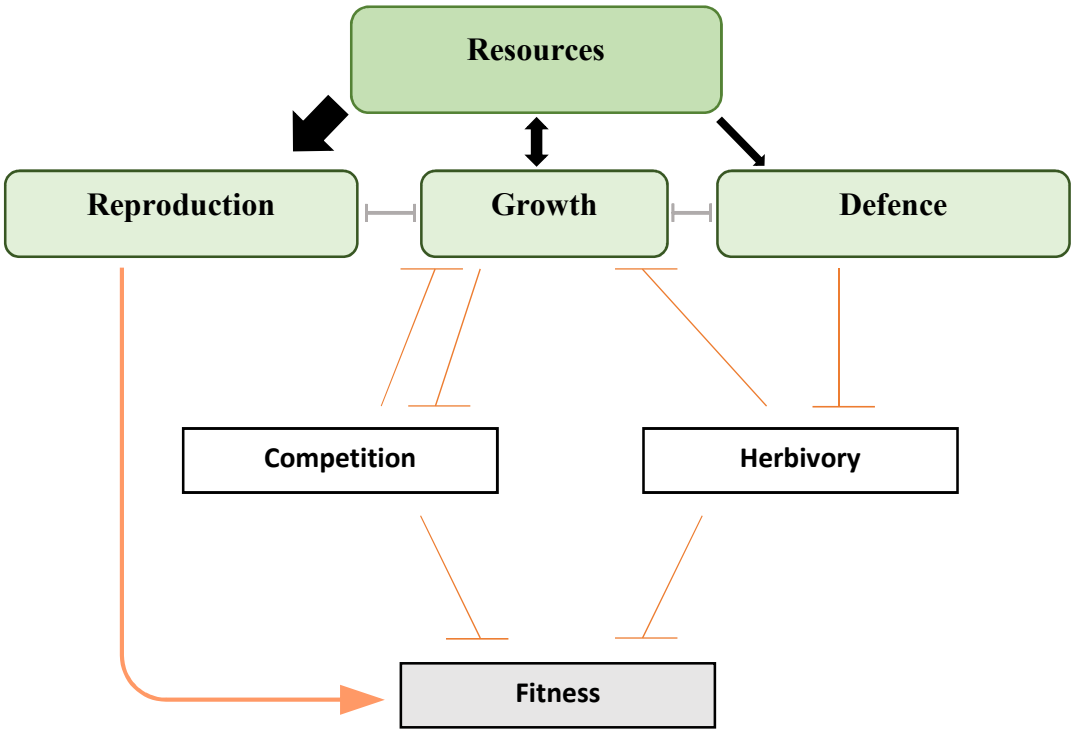


Figure 1. Graphical representation of fitness cost associated with resource allocation in plants (Zust and Agarwal, 2017). Fitness in plants is facilitated by the combined action of allocation of available resources to different life functions (*black arrows*), resource-based trade-offs (*Grey arrows*) and ecological interactions (*Orange arrow and lines*). Positive and negative effects are represented using arrows and lines with bars at the end respectively. The size of the arrow represents relative amount of the resource allocated. Growth, defense, and reproductive functions will ultimately trade-off however equal allocation of resources to any two of the three functions can also yield positive results. The resources allocated to growth maybe returned to the resource pool so that they are reallocated to other functions based on the need of the plant and a higher growth rate can reduce the negative effect of any competition. Resource allocation to defense functions is diffuse, however overall allocation of resources exclusively to defense can have negative impact on its survival.

According to a study by Rahardja and Whalon (1995), the process of acquiring resistance has led to a longer larval development period with underweighted larvae, a shorter oviposition period, smaller eggs, and decreased fertility in the *Bt*-resistant insects.

Zhang *et al.*, (2015) studied the fitness cost in chlorpyrifos-resistant *Plutella xylostella* to temperature stress and reported that there was a significant fitness cost of insecticide resistance on insect's toxicological, physiological, and biological performances. At higher temperatures, the susceptibility of the resistant insect to chlorpyrifos was found to be increased. Gene expression analysis was done on the insect after heat treatment and showed that the expression of *hsc70* had no significant upregulation in pupae and adults. However, *hsp70* and *hsp90* were highly expressed in both pupae and adults. Although the susceptible strain had lower expression of both *hsp70* and *hsp90*, the expression was higher than that of the resistant strain.

2.7. Temperature stress and heat shock proteins

Temperature is one of the most important environmental stresses affecting the distribution and development of any organism. Insects, due to their high fecundity and different morphological characteristics can be seen in a wide range of climatic conditions but their survival at higher temperatures can be very little. The metabolic rate of any insect is dependent upon the external temperature due to the exothermic nature of the insects. This knowledge has been exploited in the case of post-harvest quarantine procedures, where rapid changes in the temperature can trigger a wide range of metabolic responses as found in *Cochliomyia macellaria* larvae, in which increased production of polyphosphates and polyols were found as the insect changes to anaerobic metabolism upon stress (Meyer, 1978).

Temperature can also affect the insect's respiration, proper functioning of different organ systems including the endocrine and nervous system. In honeybees, the behavioral performance and physical structure of adults can be influenced by the effect of temperature at pupal stage (Tautz *et al.*, 2003).

Tolerance against thermal stress is essential for insects to complete their life cycle, exploring their habitat, and surviving extreme conditions (Hoffmann *et al.,* 2003; Chidawanyika and Terblanche, 2011). Insects upon exposure to any stress conditions tend to exhibit changes in their physiological characteristics, morphology, life history traits and behavior (Duman,2003; Kelly *et al.*, 2012; Lu *et al.*, 2014). Production of heat shock

protein (HSPs) is one of the most widely studied strategies adopted by insects in response to heat stress (Feder & Hoffmann, 1999).

2.7.1. Heat shock proteins

Heat shock proteins (HSPs) are ubiquitous proteins and are known for their responsive action towards multiple stresses that include extreme temperature, anoxia, desiccation, heavy metal toxicity, hypertonic stress, ethanol, and other contaminants (Lui *et al.*, 2013; Chen *et al.*, 2015). They also play an important role in most organisms as "chaperonin proteins", which aid in the folding and assembly of polypeptides, maintaining the regular proteins in their folded state, translocation of nascent proteins across cell membranes, minimizing the aggregation of non-native proteins and initiate the removal and degradation of any aggregated or non-native proteins from the cells (Currie and Tufts, 1997; Lewis *et al.*, 1999; Nadeau *et al.*, 2001; Sejerkilde *et al.*, 2003).

In the case of temperature stress, the most important coping mechanism is involved in minimizing the aggregation of the proteins and removal of any denatured proteins. In addition to providing protection, this family of proteins plays an important role in other developmental processes including, embryonic development, cell cycles, cell differentiation and hormonal stimulation (Huang *et al.*, 2009; Jiang *et al.*, 2012).

There exist different families of HSPs that are reported in various organisms and those found in insects are grouped into several families based on their homology and molecular weight, which includes HSP60, HSP70, HSP83, HSP90, HSP100 and small heat shock proteins (shsp).

Goodman *et al.* (2012) in a study on *Tribolium*, reported that the expression of *hsp* genes was recorded the highest at 20 min incubation and decreased until 60 min, where the expression was found to be similar to the control used. While another study concluded that the expression was lower at 20 min incubation and the expression increased at 40 and 60 min (Garcia-Reina *et al.*, 2017).

Sang et al (2012) studied the effect of UV-A exposure on different hsp gene (*hsp27, hsp68* and *hsp83*) expression and reported that the genes were upregulated in all the cell lines used in the study. The study also reported that all three genes showed a similar response to the stress exposed, with *hsp27* reported to be more sensitive than the other two genes studied.

Yi *et al.* (2018), characterized six different heat shock protein genes in *Trichogramma chilonis*, which included *Tchsp10, Tchsp21.6, Tchsp60, Tchsp70, Tchsc70.3* and *Tchsp70*. This study also showed that during the development, the expression of *Tchsp10, Tchsp21.6* and *Tchsp60* decreased in pupal and adult stages, *Tchsc70* was expressed highest in the larval stage and lowest in the pupal stage. However, the expression of *Tchsp70* and *Tchsp90* was the largest in adult stages.

Lu *et al.* (2018) reported that applying high temperature to the *T. castaneum* larval population showed elevated adaptation to heat stress. They also revealed that some important processes were involved in the adaptation of larvae to elevated temperatures which included unsaturated fatty acid biosynthesis and processing of proteins in the endoplasmic reticulum.

The heat induced expression of *hsp* genes vary depending on the organism. For instance, *hsp70* and *hsp90* are highly expressed in *Empoasca onukii* under both heat and cold treatment (53), whereas in *Thitarodes pui,* there is an increased expression of *hsp90* than *hsp70* (Zou *et al.*, 2011). Species-specific differential expression has been widely reported too.

2.7.1.1 Hsp70

Among all the hsps synthesized in the cell, the hsp70 protein family is reported to be the most prominent response under any stress (Nadeau *et al.*, 2001). Traditionally, *hsp70* genes are divided into two groups, (i) genes that are induced whenever exposed to any stressful condition and return to their normal level of expression when the stress condition is absent, (ii) these are not stress-induced or constitutively expressed, they are also called as heat shock cognates (HSC) (Lang *et al*., 2000; Qin *et al.*, 2003). In eukaryotes, HSC in large quantities is expressed under non-stressful conditions. The cells contain multigene families that encode different 70-kDa proteins that are related but have different regulations and locations for action. These proteins include stress- induced HSP72 and constitutive HSC73 (Matranga *et al*.,2000).

Lang *et al.* (2000) suggested that the *hsp70* genes in the African clawed frog, *Xenopus laevis* (Daulin) are not induced by heat stress until the mid-blastula transition, while constitutive *hsc70* mRNA can be detected even in the cleavage stage of the embryo, which also suggests the transcript is of maternal origin. Ritossa and Vonborstel (1964)

first documented heat shock protein expression in *Drosophila melanogaster* after subjecting to heat stress was done in 1964.

Mahroof *et al.* (2005) studied temperature-induced changes in protein expression in different life stages of *T. castaneum* and reported that the young or first instar larva is the most heat tolerant stage to elevated temperature than other life stages. This study also suggested that there is an independent regulation of the expression depending on the stages of the insect. They also reported that in eggs, the heat induced-HSP70 was not activated and thereby no HSP70 protein was synthesized. However, the constitutive HSC70 was expressed in high levels in the eggs.

In *Drosophila*, the first instar larva was found to be most heat tolerant and the heat induced HSP70 expression was said to be contributing to the heat tolerance, however the fitness of the larva and their larval development to adulthood was affected drastically (Krebs *et al.*, 1998).

2.7.1.2. Hsp60

Heat shock proteins of 60 kDa are prevalent in prokaryotes and eukaryotes and are necessary for native protein folding, translocation, and assembly. As molecular chaperones, HSP60s work with other chaperones Hsp10/GroES, Hsp70) to carry out their distinct roles (Hartl and Hayer-Hartl, 2002). HSP60s, like the other HSP families, are distinguished by their capacity to recognize proteins with unusually exposed hydrophobic residues and by their capacity to form persistent inactive aggregates upon interaction (Hinault *et al.*, 2006; Natalello *et al.*, 2013).

In *C. chilonis*, *Cchsp60* is triggered in case of cold stress and insensitive to heat stress (Pan *et al.*, 2018), while in *P. puparum*, the *hsp60* gene reaches its maximum expression at even 32℃ (Wang *et al.*, 2012).

2.7.1.3. Hsp90

The members of the hsp90 family are homodimers that share a common structural plan and their molecular weight ranges from 82 to 94 kDa. This family of proteins is reported in every species except in Archaea (Kim *et al.*, 1998; Large *et al.*, 2009). Structurally, they consist of an ATP-binding domain at N-terminal (25kDa), a C-terminal dimerization domain (12 kDa) and a middle domain (35 kDa) (Young *et al.*, 2001; Meyer *et al.*, 2003; Street *et al.*, 2011; Marzec el al., 2012). Different isoforms of HSP90 exist in

eukaryotes, as in the case of *D. melanogaster* and *Caenorhabditis elegans*, where only one cytosolic homolog of HSP90 exist, whereas in the case of mammals and yeast cytosol there exist two homologs that are closely related (Johnson, 2012).

This family of proteins is believed to be involved in protecting signaling kinases and receptors of steroid hormones. In *Drosophila*, Hsp90 is reported to be involved in embryogenesis, oogenesis, and spermatogenesis (Song *et al.*, 2007; Pisa *et al.*, 2009) while in *Caenorhabditis elegans*, these proteins were found to play an important role in their cell cycle (Inoue *et al.*, 2006).

In addition to the cytosolic groups, there exist another group which is endoplasmic reticulum (ER) based homologs of HSP90 which are also referred to as 94-kDa glucose-regulated protein, gp96, endoplasmin, or GRP94. These homologs have an ER- targeting motif (KDEL) in their C-terminal and an additional signal peptide in the N-terminal apart from which both the sequence of ER-based and cytosolic HSP90 homologs show high similarity (Pelham, 1989; Marzec *et al.*, 2012). There have been reports of HSP90 homologs in mitochondria and chloroplast which show less similarity but have specific functions in the cell organs. High-temperature protein G (HTPG) specific for prokaryotes which are found in bacterial cytosol are also HSP90 homologs (Chen *et al.*, 2006).

ER- based HSP90 was reported to be involved in the maturation of proteins that were destined for the cell surface or export. They are also found to be vital in case of immune reactions, both in innate and adaptive immune systems (Yang *et al.*, 2007; Tramentozzi *et al.*, 2011). They are also pivotal during the early developmental stages in *C. elegance,* mouse, and fly. The HSP90 orthologous protein mutant larva in fly was found to have defects in their midgut epithelium (Wanderling *et al.*, 2007; Maynard *et al.*, 2010).

2.7.1.4. Hsp100

Bacteria, protozoa, fungi, and plants all are reported to be carrying Hsp100 chaperones, but they are not found in animals or humans. Among this family, the best-studied group of proteins is Hsp104, which includes the bacterial ClpB and its homolog identified in yeast. These chaperons can form hexamers of ring-shaped structure when there is ATP present, with a central channel (pore) which is wide enough to accommodate unfolded polypeptide chains. (Lee *et al.*, 2003; Zolkiewski *et al.*, 1999; Akoev *et al.*, 2004; Lin and Lucius, 2015).

They are involved in protein quality control thereby maintaining proteostasis (homeostasis) in bacterial cells, aiding their survival during stressful conditions. These are also involved in reactivating protein aggregation. Certain proteins belonging to these families are reported to be responsible for inter-cellular replication and virulence (type VI secretion system, T6SS) in bacterial pathogens ensuring the survival of the pathogen in different conditions (Capestany *et al.* 2008; Frees *et al.*, 2008; Meibom *et al.*, 2008; Alam *et al.*, 2018)

These groups of proteins due to their unique disaggregation activity, were postulated to be a potential tool for the therapy of different protein aggregation diseases in humans such as Alzheimer's disease, Huntington's disease, or Parkinson's disease. Since there is an absence of these protein homologs in humans and other animal species, these are now used as a target for antimicrobial therapies (Erives *et al.*, 2015).

2.4.1.5. Small heat shock proteins (shsps)

Similar to other heat shock proteins, this family of proteins is also involved in abiotic stress responses. They have a molecular weight of around 12 to 42 kDa, making them the smallest member among the heat shock chaperon proteins (Waters, 2013). These proteins due to their molecular weight are also referred to as Hsp20s (Waters and Vierling, 2020). Structurally they differ slightly from other families which are highly conserved. These proteins possess diversified N- terminal and a highly conserved C-terminal. The C-terminal contains an α- crystalline domain (ACD) also called the Hsp20 domain of shsps. They are therefore also termed α-crystalline proteins (Haslbeck *et al.*, 2005; El-Gebali *et al.*, 2019).

They play an important role in both biotic and abiotic stress response in plants, processing of protein aggregation and assembling of nascent proteins. They also combine with other Hsps (Hsp70 and Hsp100) as molecular chaperons binding to denatured proteins (Horwitz, 1992; Liberek *et al.*, 2008; Stengal *et al.*, 2010; Rutsdottir *et al.*, 2017; Yang *et al.*, 2017).

In pepper, CaHsp25.9 and CaHsp16.4 proteins were reported to be involved in heat, drought, and salinity stress tolerance (Feng *et al.*, 2019), while in *Malus sieversii*, MsHsp16 activated signaling pathway in response to temperature stress (Yang *et al.*, 2017). In tomatoes, these proteins are reported to protect the plants from chilling (Ré *et*

al., 2017). Other plant species in which these proteins were reported to include *Oryza sativa* (OsHsp18 and OsHsp18.2) (Kaur *et al.*, 2015; Kuang *et al.,* 2017), *Arabidopsis* (AtHsp21), *Triticum aestivum* (TaHsp26) and *Camellia sinensis* (CsHsp25.9) (Khurana *et al.*, 2013; Sedaghatmehr *et al.*, 2016; Wang *et al.*, 2017). Apart from involvement in thermotolerance, these proteins are reported to be providing protection against heavy metal stress in *Closterium ehrenbergii* (sHsp10 and sHsp17.1) and protection against oxidative stress in *Medicago sativa* (MsHsp17.7). (Li *et al.*, 2016; Abassi *et al.*, 2019).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study, "Temperature induced changes in the biology and heat shock protein gene expression in malathion resistant red flour beetle," was done in different stages. Insect rearing and bioassay studies were carried out in Pesticide Residue Laboratory, Department of Entomology, College of Agriculture, Vellanikkara. The molecular biology works pertaining to the study were carried out in the laboratories of Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, and gene expression studies were done at Central Instrumentation Lab in Kerala Veterinary and Animal Science University, Mannuthy from 2019-2021.

3.1 Chemicals, plasticware, and instruments

The chemicals needed throughout the study were purchased from Sigma-Aldrich, HiMedia, Thermo Fisher Scientific, and Merck India Ltd. All the chemicals used in this study were pure and certified. For RNA isolation, chemicals like TRI reagent (Sigma Aldrich), RNAzap (Sigma Aldrich), Chloroform (Merck India Ltd.) and Ethanol (HiMedia) were used. RevertAid first strand cDNA synthesis Kit procured from Thermo Fisher Scientific was used for first strand cDNA synthesis. Plasticware and glassware used were procured from Tarsons India Ltd. and Borosil, respectively. Primers needed were synthesized with Sigma Aldrich Pvt. Ltd.

Food-grade plastic jars were used for rearing insect cultures. Malathion (certified reference material) needed for bioassay was procured from OMC Chemicals and Allied Chromatography Pvt Ltd. List of different instruments used throughout the study are given in Table 3.

Table 3. List of instruments used during different stages of the study

Sl. No.	Steps involved		Instrument	Manufacturer
1	Temperature treatment	20°C	T100™ Thermal cycler	Bio-Rad
		35°C	SI-600	Lab Companion
		45°C	iTherm D150-2	Neuation
		60°C	iTherm D150-2	Neuation
2	Total RNA isolation			
	i. Centrifuge		Model 3500	Kubota

	ii. Nanodrop	Genova Nano	Jenway
3	Gel electrophoresis		
	i. Gel running unit	PowerPac™ 300	Bio-Rad
	ii. Gel documentation	Molecular Imager® Gel Doc™	Bio-Rad
4	First strand cDNA synthesis		
	i. Spinner	Spinwin MC-100	Tarsons
	ii. Incubation	T100™-Thermal cycler	Bio-Rad
5	RT-qPCR	StepOnePlus™	Applied Biosystems

3.2 Experimental organism

This study used two different strains of red flour beetle, *Tribolium castaneum* (Tenebrioniidae: Coleoptera), malathion resistant and susceptible strain. Malathion resistant strain was collected from the Food Corporation of India (FCI) godown, Angamali. A susceptible strain, obtained from Division of Entomology, IARI, New Delhi maintained in the Department of Entomology, College of Agriculture without any pesticide exposure for more than 30 years was used in the study. From the initial culture, mass culturing was done to obtain insects of uniform size and age for various experiments.

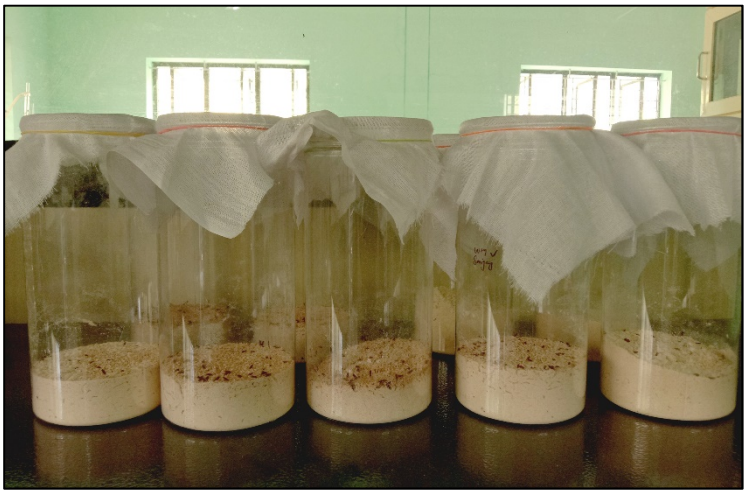
3.3 Mass culturing of insect

The population collected from FCI, Angamaly, and IARI strain was mass cultured in plastic jars with wheat (95%) and yeast (5%), and was maintained at 28°C with a relative humidity of 70% (Padin *et al.,* 2002). The wheat flour was sterilized at 50°C for 6 hours in hot air oven. A total of 15- 20adult pairs were released in each jars. The insects were then subcultured at regular intervals to obtain insects of uniform age and size.

3.4. Biology of insects

Insect biology was studied from 2019-2021 to have an accurate and broader understanding of the duration of different life stages and their life cycle.

(a)



(b)

Plate 1: (a) Rearing of malathion resistant and susceptible strains of *Tribolium castaneum.* (b)Rearing media: 95g wheat flour with 5g yeast

3.4.1 Observations recorded

The following observations on red flour beetle to study their life cycle were taken:

3.4.1.1 Incubation period: time taken from oviposition to larval emergence.

3.4.1.2 Larval period: Starting from hatching of the eggs till the day of pupation. Different larval instars and their change in colour were also observed.

3.4.1.3 Pupation period: Number of days from final moulting of the last instar larva to the emergence of adult beetle. The colour of the pupa was also recorded.

3.4.1.4 Adult emergence: The emergence of adults occurred from the pupa.

3.5. Temperature treatment

The different life stages of the beetles, namely larva, pupa, and adults, were subjected to three different temperatures i.e., 20℃, 35℃, 45℃ and 65℃ for 1 h. After the treatment, the insects were evaluated for their susceptibility to malathion after the heat treatment. Total RNA was also isolated from the insects to study heat shock protein gene expression.

3.6. Total RNA isolation

Following the heat treatment, total RNA was isolated from each life stage after allowing a recovery period of 1h in room temperature. The fifth instar larva (21 days old), three days old pupa, and 17 days old adults were selected for heat treatment followed by RNA isolation. All the plastic wares and materials involved in RNA isolation were treated with DEPC (Sigma Aldrich), left overnight, and double autoclaved.

Protocol:

1. The insect samples, 100 mg of adult, larva, and pupa after heat treatment, were taken in a 2 ml test tube and homogenized thoroughly in 1 mL Tri reagent using micro-pestle. For better yield, 500µL of Tri reagent was first added, and upon thorough homogenization, the rest 500µL was added. The contents were then incubated for 10 minutes at room temperature.
2. Then 200µL cold chloroform was added to separate the phases. After adding chloroform, the contents were shaken vigorously for 15 seconds and incubated at

room temperature for 2-3 minutes. This step was repeated to remove excess protein from the samples.

3. The contents were then centrifuged at 12000xg at 4℃ for 15 minutes. There was visible phase separation, with a pink bottom phase and a clear aqueous phase.
4. The clear top fluid was removed and transferred to a new labeled tube. The tube with all the protein and other sediments were discarded.
5. To the clear solution, 500µL of cold isopropanol was added. It was then incubated for 15 minutes at room temperature. Then the contents were centrifuged at 12000xg 4℃ for 10 minutes. The RNA was pelleted at the bottom. The supernatant was then discarded.
6. The pellet was then washed with 75% ethanol, followed by a brief vortex and a short spin at 7500xg for 5 minutes at 4℃. The step was repeated twice.
7. After the wash, ethanol was removed from the tube and dried at room temperature.
8. The pellets were then resuspended in 30µl nuclease-free water.
9. The tubes were labeled and stored at -80℃ for long-term storage.
10. The quality and quantity of the RNA isolated were determined through nanodrop and gel electrophoresis.

3.8. First strand synthesis of complementary DNA (cDNA)

The first strand was synthesized from each RNA sample using RevertAid cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturers protocol.

Protocol:

* The components provided in the kit were thawed, followed by a brief mixing and centrifugation, and stored in ice.
* The template RNA (4µl), Oligo dT primers (1µl), and then nuclease-free water (7µl) was added to make up the volume to 12µl.
* The tubes were then incubated at 65℃ for 5 minutes.
* Immediately after incubation, the tubes were chilled in ice, followed by addition of 5X reaction buffer (4µl), RiboLock RNase inhibitor (1µl), 10mM dNTP mix (2µl), and RevertAid M-MuIV Reverse Transcriptase (1µl).
* The tubes were mixed gently and centrifuged briefly.

* Tubes were then incubated at 42℃ for 60 minutes, followed by incubation at 70℃ for 5 minutes.
* After the incubation, the samples were labelled and stored at -80℃.

3.9. Gene expression studies

The cDNA synthesized from each RNA sample was used for expression analysis using RT- qPCR. The gene expression of *Hsp70* was analyzed with β-*actin*, as the reference gene. This step was performed using StepOnePlus™ PCR system, and the software used was StepOneV2.2.2. A comparative C_T ($\Delta\Delta C_T$) program in the software was used to analyze the gene expression. The primers for the target gene used for amplification were designed using Primer3 software after obtaining the nucleotide sequence for *hsp70* mRNA (Genbank ID: MK0004391) from NCBI. The reaction mixture was prepared for the PCR amplification with the components mentioned as in table 4.

Table 4. Preparation of mix used for gene expression analysis

Sl No.	Component	Volume
1	SsoAdvanced™ Universal SYBR® Green Supermix	10 µl
2	cDNA template	5 µl
3	Forward primer	1 µl
4	Reverse primer	1 µl
5	Nuclease free water	3 µl
	Total volume	**20 µl**

Triplicates of each diluted cDNA sample were loaded into the PCR strips with both sets of primers for *Hsp70* and β-*actin* and non-template control (NTC) for each gene. After loading the samples, they were amplified using the following programme.

Table 5. Thermal profile of the gene expression analysis

Step	Stage	Temperature (°C)	Time (Sec.)
1	Polymerase activation and DNA denaturation	95	30
2	Denaturation	95	15
3	Annealing/ Extension	60	30
4	Melt Curve Analysis	95 60 95	15 60 15
Denaturation (step 2) and annealing (step 3) were repeated for 45 cycles			

3.9.1. Calculation of fold change

C^T values that were recorded after the amplification was further used to calculate ΔC^T and $\Delta\Delta C^T$ of each of the samples. They were calculated using the following formula.

$$\Delta C^T = C^T \text{(gene of interest)} - C^T \text{(housekeeping gene)}$$

$$\Delta\Delta Ct = \Delta C^T \text{ (Treated)} - \Delta C^T \text{(Untreated)}$$

Here, the treated samples were insect samples subjected to different temperature stress, and the untreated samples were those insect samples not exposed to any heat stress. The fold change in expression can be calculated using the formula,

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

3.10. Bioassay

The susceptibility of both IARI and Angamaly strains after heat treatment were evaluated against technical grade malathion. The residual film method was followed to evaluate the susceptibility. A stock solution of 1000ppm was prepared by dissolving an appropriate amount of malathion in acetone. Required concentration was obtained through serial dilution.

The concentration of insecticides used in the bioassay was arrived after conducting a preliminary bioassay using commercial-grade malathion (Sigma Aldrich). Five concentrations were involved in the bioassay for each sample, including control, each of which was triplicated. From the prepared concentrations, one milliliter of the insecticide was pipetted out and added to petri plates of 9mm diameter. In the case of

control, one milliliter of acetone was added. The plate was then rotated so that a thin film of uniform thickness of the insecticide was obtained over the plate and the plate was air dried. Once dried, ten adults of the insects (17±2 days old) were introduced into each plate. The insects were exposed to different temperatures for one hour and given a recovery period of another hour before the bioassay. The mortality of the insects was recorded after 24h. Moribund insects were also counted as dead.

The number of dead insects were taken, and the LC_{50} value was then calculated for each population using Polo PC software. Whenever required, mortality was corrected using Abbott's formula (Abbott, 1925). The resistance ratio was then estimated using the given formula.

$$Resistance\ ratio = \frac{\text{LC50 of the resistant } strain}{\text{LC50 of the susceptible strain}}$$

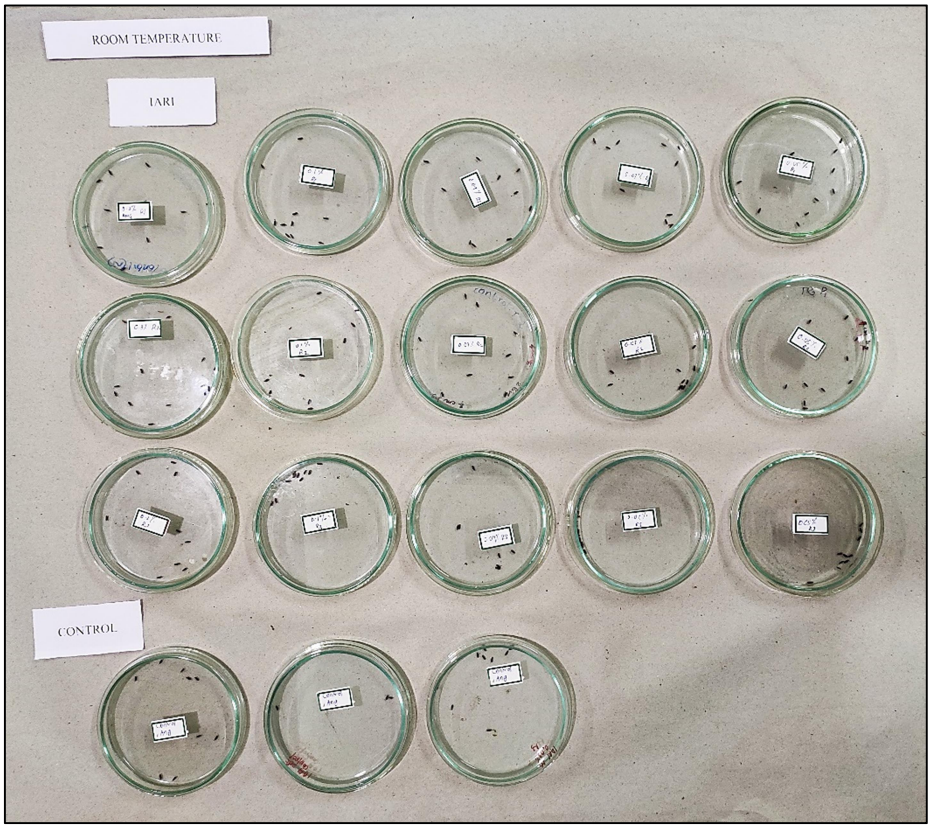


Plate 2. Residual film bioassay of adults using malathion

RESULTS

4. RESULTS

The results of the study entitled, "Temperature induced changes in the biology and heat shock protein gene expression in malathion resistant red flour beetle (RFB), *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae)" are presented here in this chapter.

4.1. Rearing of insects

The test insects, malathion resistant (Angamaly) strain was collected from FCI godown, Angamaly and susceptible strain (IARI) from Division of Entomology, IARI and reared in wheat flour (95%) with yeast (5%). Insects of uniform size and age were collected and used for various experiments.

4.2. Biology of insects

The life cycle of both the test insect strains were studied during the period. The data provides insight into the life cycle of both malathion resistant and susceptible strains of RFB. The total life cycle of the insect was found to be completed in three to four weeks. The duration of each of the life stages recorded is given in Table 6.

Table 6. Duration of different life stages of malathion resistant and susceptible strain.

Sl. No.	Life stage of insect	Duration of each stage (in days)	
		IARI strain	Angamaly strain
1	Egg	3.4 ± 0.52	4.6 ± 0.52
2	Larva	24.6 ± 0.84	26.3 ± 0.67
3	Pupa	4.4 ± 0.7	5.2 ± 0.79
Mean of 5 observations			

4.2.1. Adult

Adults of both the malathion resistant and susceptible strains were identical in size. They were both reddish brown colored, and their life period was found to be 6-7 months. Both strains have completed their life cycle in 4-5 weeks.

4.2.2. Egg

Eggs were creamy white in color and were found sticking on the wheat flour. The incubation period for eggs to hatch into larva was recorded as 3.4 ± 0.52 days in the case of susceptible strain and 4.6 ± 0.52 days in malathion resistant insects.

4.2.3. Larva

After the incubation period, eggs hatch into yellowish white slender larvae with segmented body and three pairs of legs. The larval period in susceptible strain was found to be 24.6 ± 0.84 days, while it was 26.3 ± 0.67 days in the case of the resistant strain.

4.2.4. Pupa

Pupae were whitish in color and naked. Pupal period in the susceptible strain was found to be 4.4 ± 0.7 days and 5.2 ± 0.79 days in the case of resistant strain.

4.3. Effect of temperature on the mortality of *Tribolium castaneum*

4.3.1. Temperature treatment

The larval, pupal, and adult stages of both Angamaly and IARI strains were exposed to different temperatures (20℃, 35℃, 45℃ and 60℃) for 1h. The life stages were then studied for any change in the life cycle.

4.3.2. Effect of temperature on mortality of adults of IARI and Angamaly strains

Ten adults (17±2 days old) of Angamaly and IARI strains after temperature treatment were studied for their mortality. The adults of both Angamaly and IARI strains showed no mortality after 1h heat treatment at 20℃, 35℃, and 45℃. However, the mortality was 100.0 per cent when the adults were treated at 60℃ (Table 7). The results indicate that there was no adult mortality when exposed for 1h at various temperatures, except at 60℃.

Table 7. Mortality of Angamali and IARI adults at different temperature treatments

Sl. No.	Temperature (℃)	Percent mortality of the insect after 1 hr recovery	
		IARI	Angamaly
1	20	0.0^{b} (0.0)	0.0^{b} (0.0)
2	35	0.0^{b} (0.0)	0.0^{b} (0.0)
3	45	0.0^{b} (0.0)	0.0^{b} (0.0)
4	60	100.0^{a} (90.0)	100.0^{a} (90.0)
Values in the parenthesis are arc sine transformed Values followed by the same alphabets did not differ significantly at $p < 0.05$			

4.3.3. Effect of temperature on mortality of larvae of IARI and Angamaly strains

Ten larvae each of Angamaly and IARI strains after heat treatment were studied for their mortality. The mortality of both Angamaly and IARI strains were following a similar trend, which was lowest at 35°C (4.0% for IARI and 6.0% for Angamaly). The mortality increased when the temperature increased to 45°C (20.0% for IARI and 34.0 for Angamaly) and 60°C (100.0% mortality for both IARI and Angamaly). When the temperature was lowered to 20°C mortality was 12.0% for IARI and 14.0% for Angamaly strains. The results showed that temperature stress affected the larval mortality, and the larvae of resistant strain (Angamaly) was found to have higher mortality at all the temperature treatments compared to its susceptible (IARI) counterpart (Table 8.).

Table 8. Mortality of larval stage at different temperature treatments

Sl. No.	Temperature (°C)	Percent mortality of the insect	
		IARI	Angamaly
1	20	12.0 [c] (20.06)	14.0 [c] (21.69)
2	35	4.0[d] (7.37)	6.0 [d] (11.06)
3	45	20.0[c] (26.27)	34.0[b] (35.62)
4	60	100[a] (90.0)	100 [a] (90.0)
Values in the parenthesis are arc sine transformed Values followed by same alphabets did not differ significantly at $p < 0.05$			

4.3.4. Effect of temperature on adult emergence in IARI and Angamaly strains

After temperature exposure, the adult emergence from pupae of both IARI and Angamaly strains were studied. The adult emergence at room temperature (25±0.5°C and 81± 5% R.H) for both IARI and Angamaly was 80.0% and 83.3%, respectively. When the pupae were exposed to temperature stress, significant changes were observed in adult emergence. The emergence was found to be 76.67% and 73.33% for IARI and Angamaly strains when exposed to 20°C, respectively. The percentage then dropped to 73.33 and 66.67 at 35°C and 53.33 and 46.67 at 45°C for IARI and Angamaly strains, respectively. No adult emergence was observed at 60°C treatment. The emergence was found to be reduced with an increase in the temperature exposed, indicating that the sudden temperature exposure affected the adult emergence. (Table 9).

Table 9. Effect of temperature treatment on adult emergence

Sl. No.	Temperature (°C)	Adult emergence (%)	
		IARI	Angamaly
1	Room temperature	80.0[a] (66.15)	83.3[ab] (63.44)
2	20	76.67[ab] (61.22)	73.33[bc] (59.00)
3	35	73.33[bc] (59.00)	66.67[c] (54.78)
4	45	53.33[d] (46.92)	46.67[d] (43.08)
5	60	0.0[e] (0.0)	0.0[e] (0.0)
Values in the parenthesis are arc sine transformed. Values followed by same alphabets did not differ significantly at $p < 0.05$			

The temperature exposure given to different stages showed that the pupal stage was affected the most, followed by the larval stage. The adult stage, however, was never affected by the stress induced. Comparing the strains, the IARI (malathion susceptible) strain was the least affected strain compared to Angamaly (malathion resistant) strain.

4.4. Effect of temperature on *Tchsp70* gene expression in malathion resistant and susceptible strains of *T. castaneum*

4.4.1. Temperature treatment

Step followed as in 4.3.1

4.4.2. Total RNA isolation

Total RNA was isolated from larval, pupal, and adult stages of both IARI and Angamaly strains after heat treatment, each using TRI reagent (Sigma Aldrich). The total RNA isolated was then analyzed for its quality and quantity.

4.4.3. Quality and quantity analysis of isolated total RNA

The total RNA isolated was electrophoresed on 1.2% agarose gel in 1X TAE buffer prepared. The gel yielded intact bands in all the samples. The concentration and purity of the isolated RNA were estimated using Genova Nano (Jenway) spectrophotometer. The instrument recorded a concentration between 543.8 and 1872.0 ng/µl and the A260/A280 ratio was between 1.81 and 1.98 indicating the isolated RNA to be pure. The concentration and purity of each insect sample are given in Table 10.

Table 10. Quantitative analysis of total RNA isolated from different insect samples

Sl. No.	Life stage of the insect	Strain	Temperature (°C)	Concentration (ng/µl)	A260/A280
1	Adult	IARI	20	678.0	1.87
2			35	543.8	1.89
3			45	1094.0	1.86
4			60	985.3	1.83
5		Angamaly	20	1172.9	1.98
6			35	910.2	1.81
7			45	1286.4	1.98
8			60	709.8	1.89
9	Larva	IARI	20	1347.0	1.97
10			35	1330.2	1.86
11			45	980.6	1.93
12			60	977.9	1.82
13		Angamaly	20	1872.0	1.94
14			35	1130.9	1.88
15			45	1340.2	1.98
16			60	589.6	1.93
17	Pupa	IARI	20	705.0	1.89
18			35	639.6	1.92
19			45	980.6	1.96
20			60	1245.0	1.81
21		Angamaly	20	1306.0	1.84
22			35	1214.0	1.92
23			45	1245.2	1.89
24			60	1246.0	1.86
25	Adult	IARI	RT*	1451.7	1.92
26	Larva		RT*	1404.1	1.86
27	Pupa		RT*	1301.5	1.93
***RT – insect samples without any exposure to any temperature treatment**					

4.4.4. Quantitative and quality analysis of first strand cDNA samples synthesised

The cDNA samples synthesized were analysed using Genova Nano spectrophotometer and recorded the concentration ranging between 943.9 and 2177.3 ng/µl. The A260/A280 ratio was between 1.56 and 1.79. The concentration and purity of all the cDNA samples recorded are given in Table 11.

Table 11. Concentration of cDNA synthesized

Sl. No.	Life stage of insect	Strain	Temperature (°C)	Concentration (ng/µl)	A260/A280
1	Adult	IARI	20	1626.0	1.63
2			35	1597.2	1.69
3			45	1675.8	1.68
4			60	1693.5	1.66
5		Angamaly	20	2177.3	1.67
6			35	1544.0	1.54
7			45	1691.3	1.69
8			60	1454.2	1.56
9	Larva	IARI	20	1176.0	1.67
10			35	1661.1	1.66
11			45	1374.0	1.65
12			60	1938.2	1.58
13		Angamaly	20	1413.9	1.73
14			35	1351.0	1.68
15			45	1107.0	1.74
16			60	1987.0	1.69
17	Pupa	IARI	20	1749.7	1.65
18			35	1482.8	1.79
19			45	1147.7	1.75
20			60	1125.2	1.65
21		Angamaly	20	1395.2	1.76
22			35	1366.0	1.75
23			45	943.9	1.69
24			60	1469.1	1.77
25	Adult	IARI	RT*	1371.8	1.65
26	Larva		RT*	1641.4	1.65
27	Pupa		RT*	1531.2	1.63
RT*- insect samples without any temperature treatment, taken as control					

4.4.5. Primer designing for RT qPCR analysis

A pair of primers were designed for the target gene, *Tchsp70* with the sequence retrieved from NCBI (Genbank ID: MK0004391) using Primer3 software. The primers were synthesized with Sigma-Aldrich Pvt. Ltd. Primers of β -*actin* gene was used as the endogenous control. The list of primers used for analysis is given in Table 12.

Table 12. Primers used for RT-qPCR

Gene	Name of primer	Orientation	Primer	Amplicon size (bp)
Hsp70	*tchsp70*f	Forward	AACTTACGCCGACAACCAAC	188
Hsp70	*tchsp70*r	Reverse	CTTTAGCGGAGACGTTCAGG	
B-actin	*B-actin*f	Forward	TCTTCCAGCCTTCCTTCCTG	216
B-actin	*B-actin*r	Reverse	CACACAGAGTACTTGCGCTC	

4.4.6. Expression analysis using RT-qPCR

The expression analysis of *Tchsp70* gene in malathion resistant and susceptible population of red flour beetle subjected to different temperature regimes was studied using the RT-qPCR technique. The cDNA synthesized from each insect sample after heat treatment was analyzed for its relative expression using *Tchsp70* taken as the target gene and β-*actin* taken as the endogenous control (housekeeping gene). StepOne Plus (Applied Biosystems) was employed for studying the expression in different samples that were run as triplicates.

The mean cycle threshold (C_T) values of both *Tchsp70* and *β-actin* were obtained, and the relative expression was normalized with endogenous control, *β-actin* gene, using the Livak method (Livak and Schmitten, 2001). The mean C_T, ΔC_T, $\Delta\Delta C_T$, and fold expression of all the samples, taking insect samples without any temperature treatment as control, is given in Table 13.

The *Tchsp70* expression in malathion resistant strain for that of susceptible strain was calculated (Table 14). The results showed that there is an increase in expression in *Tchsp70* expression with the increase in temperature exposed. In adults, the relative expression was found to be 9.05-folds at 20℃, which increased to 73.64-folds at 35℃, and the peak expression of 171.83-folds at 45℃ and then the expression dropped to 63.90-folds at 60℃. Similarly, in larvae, the fold expression was 0.048, 0.259, 8.449, and 0.119 at 20℃, 35℃, 45℃, and 60℃ respectively. In the pupal stage, the expression followed a similar trend, 0.08-fold expression at 20℃, 9.629-fold expression at 35℃, 14.74-fold expression at 45℃, and 8.401-fold at 60℃.

Table 13. Fold expression of *Hsp70* in different life stages of malathion resistant and susceptible strains of *Tribolium castaneum* after heat treatment

Sl. No.	Strain	Temperature (°C)	Life stage	CT mean value		ΔCT	ΔΔCT	Fold expression
				Target gene (*Hsp70*)	Housekeeping gene (β-*actin*)			
1	IARI	Control*	Adult	33.95509	33.39367	0.56142	0	1
2			Larva	30.72266	36.20716	-5.4845	0	1
3			Pupa	35.10389	30.63183	4.472065	0	1
4		20	Adult	21.69063	27.91551	-6.22488	-6.7863	110.3775 (62.71-194.26)
5			Larva	18.47238	29.56182	-11.0894	-5.60495	48.66953 (25.99-91.15)
6			Pupa	23.50743	28.25104	-4.74361	-9.21568	594.5587 (214.58-1647.369)
7		35	Adult	22.15987	29.22219	-7.06232	-7.62374	197.2301 (127.05-306.17)
8			Larva	18.03289	29.86887	-11.836	-6.35148	81.65547 (24.27-274.76)
9			Pupa	26.66225	28.47861	-1.81636	-6.28843	78.16368 (33.39-182.97)
10		45	Adult	22.88458	30.02478	-7.1402	-7.70162	208.1709 (72.98-593.76)
11			Larva	20.2199	34.82968	-14.6097	-9.12524	558.4322 (213.61-1459.92)
12			Pupa	29.36413	37.70988	-8.34575	-12.8178	7220.173 (2077-25099.14)
13		60	Adult	21.06992	28.53096	-7.46104	-8.02246	260.0159 (171.13-395.08)

14			Larva	20.32182	31.19112	-10.8693	-5.3848	41.7818 (28.14-62.03)
15			Pupa	29.97358	29.26035	0.713234	-3.75883	13.53695 (6.55-27.96)
16	Angamaly	20	Adult	20.33236	29.73553	-9.40317	-9.96459	999.1743 (817.07-1221.86)
17			Larva	23.94263	30.84795	-6.90532	-1.42082	2.67738 (1.94-3.69)
18			Pupa	35.31892	36.47425	-1.15534	-5.6274	49.43293 (21.01-116.31)
19		35	Adult	18.94498	32.20969	-13.2647	-13.8261	14523.78 (6346.40-333237.75)
20			Larva	20.13508	30.33926	-10.2042	-4.71968	26.34916 (10.20-67.37)
21			Pupa	30.36413	35.44802	-5.08389	-9.55596	752.7149 (238.84-2372.26)
22		45	Adult	19.94543	34.51047	-14.565	-15.1265	35769.92 (6535.45-195776.4)
23			Larva	18.44543	35.27612	-16.8307	-11.3462	2603.423 (124.19-3410.77)
24			Pupa	23.73106	35.95859	-12.2275	-16.6996	106434 (45275.07-250208.2)
25		60	Adult	15.78851	29.24737	-13.4589	-14.0203	16615.95 (6818.68-40490.18)
26			Larva	22.88579	30.71922	-7.83343	-2.34893	5.094478 (3.64-7.13)
27			Pupa	26.77209	29.12953	-2.35744	-6.8295	113.7326 (73.01-177.15)
*Samples taken without any exposure to heat treatment								

The relative expression was found to be highest in the adult stage, followed by the pupal and larval stage. The expression was increasing from 20℃ to 45℃, where the expression was at the highest and then the values were dropping when the temperature was raised to 60℃.

Table 14. Relative gene expression of *Hsp70* in malathion resistant *Tribolium castaneum*

Life stages of the insect	Temperature (℃)	Relative expression of *Tchsp70*
Adult	20	9.052333
	35	73.63877
	45	171.8295
	60	63.90358
Larva	20	0.048119
	35	0.259773
	45	8.44914
	60	0.119441
Pupa	20	0.083142
	35	9.629983
	45	14.7412
	60	8.401641

4.5. Evaluation of susceptibility of malathion resistant strains after temperature stress

4.5.1. Temperature treatment

Ten adults of both malathion resistant and susceptible strains that are 17±2 days were selected to study the effect of temperature on malathion resistance. Temperature treatments were done as in 4.3.1. The insects were then allowed a recovery period of 1 h before bioassay.

4.5.2. Bioassay

Temperature treated adults (17±2 days old) were taken to evaluate the susceptibility of the insects to malathion using the residual film bioassay technique. The susceptibility levels were deduced from the LC_{50} values calculated by probit analysis

using Polo Plus Version 2.0. The susceptibility of different strains after temperature exposure is given in Table 15.

The Angamaly strain showed the maximum LC_{50} value (3020.0ppm) at room temperature. The LC_{50} values at 20℃, 35℃, and 45℃ were 700.0, 930.0 and 700.0 ppm, respectively. In the case of IARI strain, the LC_{50} value at room temperature was found to be 150.0 ppm, and the LC_{50} values upon temperature exposure were found to be 100.0ppm (20℃), 150.0ppm (35℃) and 240.0ppm (45℃).

Table 15. Susceptibility of Angamaly (malathion resistant) and IARI (susceptible) strains of RFB after temperature treatment

Temperature (℃)	Strain	LC_{50} (ppm) (95% fiducial limit)	LC_{90} (ppm) (95% fiducial limit)	Heterogeneity		Slope
				d.f.	χ^2	
20	Angamaly	700.0 (560.0-910.0)	1390.0 (1030.0-3530.0)	3	3.2968	0.604273
	IARI	100.0 (50.0-140.0)	300.0 (210.0-1730.0)	3	3.4713	0.492556
35	Angamaly	930.0 (830.0-1130.0)	1720.0 (1380.0-2640.0)	3	1.018	1.09190
	IARI	150.0 (90.0-250.0)	360.0 (220.0-2200.0)	3	5.7104	0.434942
45	Angamaly	700.0 (560.0-830.0)	1050.0 (870.0-1790.0)	3	4.3156	1.42874
	IARI	240.0 (160.0-430.0)	550.0 (370.0-2360.0)	3	5.7311	0.35833
RT*	Angamaly	3020.0 (2080.0-6860.0)	13030.0 (6050.0-88810.0)	3	1.907	0.190436
	IARI	150.0 (100.0-230.0)	480.0 (290.0-2180.0)	3	3.4141	0.323077

LC_{50} = Concentration of the insecticide (ppm) that gives 50% mortality: LC_{90} = Concentration of the insecticide (ppm) that gives 90% mortality

*RT: Room temperature (25± 0.5℃, 81± 5% R.H.)

DISCUSSION

5. DISCUSSION

Results obtained in the study on "Temperature induced changes in the biology and heat shock protein gene expression" in red flour beetle, *Tribolium castaneum,* Herbst. (Tenebrioniidae: Coleoptera) are examined in this chapter.

5.1. Biology of the insect

The biology and life cycle of two strains of the test insect *viz.* malathion resistant strain collected from FCI, Angamaly and susceptible strain (Division of Entomology, IARI) which has been reared without malathion exposure for more than thirty years were studied during the period from 2019-2022.

The life cycle of both strains of insect was found to be similar however the larval period of malathion resistant strain was found to be slightly longer than that of the susceptible strain. While the egg hatching period and pupal period did not show any significant changes in both strains. The result showed that the resistant strain showed a longer developmental period than its susceptible counterpart.

Slower development of immature stages was reported in the case of insects that were found to have also developed resistance against an insecticide. In case of *Ceratitis capitate*, the λ-cyhalothrin resistant strains showed an alteration in different biological parameters like the viability of pupae, longer developmental period from egg to pupa and adult weight and longevity of the insect adults when compared to their susceptible counterparts (Gulliem-Amat *et al*., 2020).

Although slower development is a method to adapt to a stress condition, this elongation of the development period further increases the chance of attack by natural enemies and therefore was found to be countering the advantage gained by the insect in the treated environment (Wilson *et al.,* 2007).

Studies conducted on *B. tabaci*, showed that phenotypic changes were exhibited by insect strains that developed thiamethoxam resistance. The adult longevity, duration of each of the lymph stages and fecundity were found to be significantly lower in thiamethoxam resistant strain than that of the susceptible (Feng *et al.,* 2009).

Nicastro *et al.* (2011) also reported that in milbemectin-resistant *Tetraninchus* urticae, the duration of stages and the duration from egg to adult female did not

significantly show any difference however the interval was shorter for the susceptible strain to that of the resistant strain. They also reported that there was a difference in the longevity of male and female adults, The longevity of susceptible females was found to be longer and in the case of males, the resistant males showed longevity.

Similarly, in acetamiprid-resistant *B. tabaci*, the nymph development period and eggs laid per female were found to be significantly lower compared to its susceptible counterpart (Basit *et al.,* 2012). However, there have also been reports of no observed cost as in the case of pyriproxyfen-resistant *B. tabaci* (Crowder *et al.,* 2009).

Skourti *et al.* (2020) studied the life cycle of *T. castaneum* exposed to chlorfenapyr with that of a control treatment and reported that there were no significant changes in the biological features. The egg hatching, larval and pupal periods for chlorfenapyr treated and untreated were found to be almost similar *i.e.,* 4.76 and 4.66, 25.71 and 25.85 and 5.26 and 5.00 days respectively.

There have been various reports of changes in life history traits as a penalty for insecticide resistance developed which are evident in this study too, where there are changes in the duration of the developmental stages.

5.2. Effect of temperature on insect biology

Several studies have reported the use of elevated temperature as an effective strategy for controlling the attack of *T. castaneum* in storage godowns. Ambient temperature plays an important role in the development of insects. Any exposure to stressful conditions can enable the insects to adapt to the change, however at the cost of changes to their biology.

In this study there is evidence for changes in the survival rate of adults, larvae and adult emergence upon temperature exposure. There were no significant changes in the mortality of the adults in both strains at 1h incubation at different temperatures. All the adults recovered (after 1h of heat treatment followed by 1h recovery) at 20, 35 and 45°C, while no adults recovered after 60°C heat treatment (Figure 2).

In the case of larvae, mortality was found to be higher for the Angamaly strain in every temperature treatment compared to the IARI counterpart. Larval mortality at 35°C was 4.0 and 6.0 % respectively for IARI and Angamaly strains, and at 20°C, mortality increased to 12.0 and 14.0 % and at 45°C, the mortality peaked at 20.0 and 34.0% for

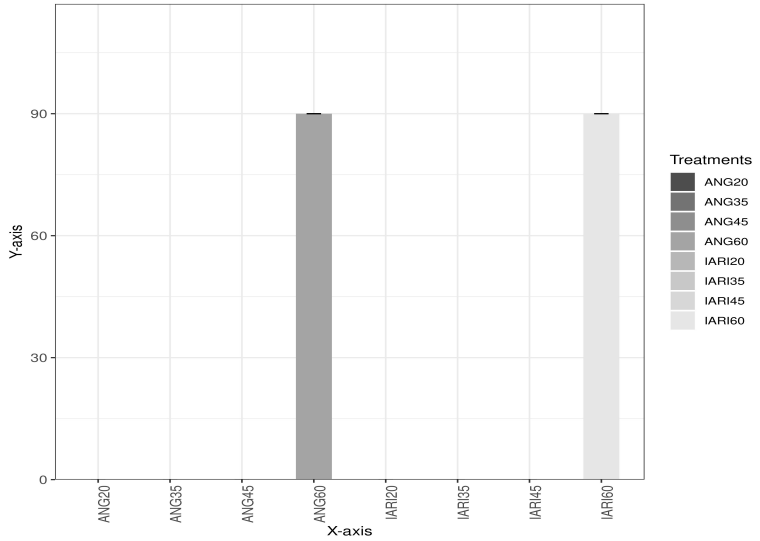


Figure 2. Effect of temperature on survival of Angamaly and IARI adults

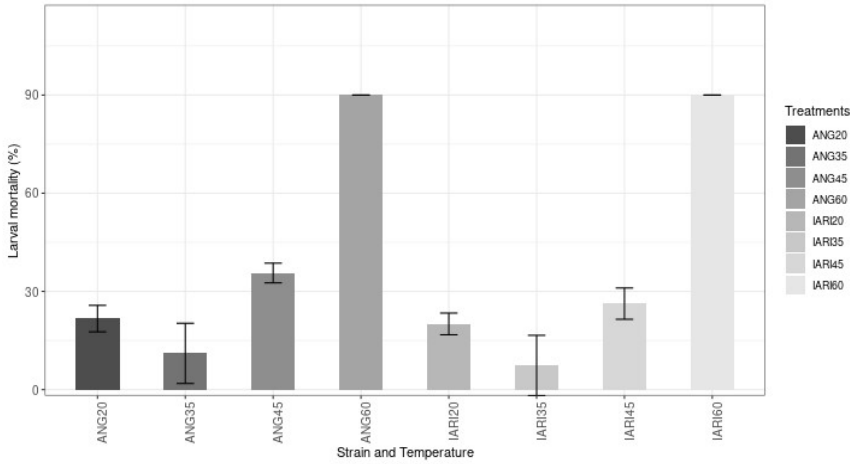


Figure 3. Effect of temperature on survival of Angamaly and IARI larvae.

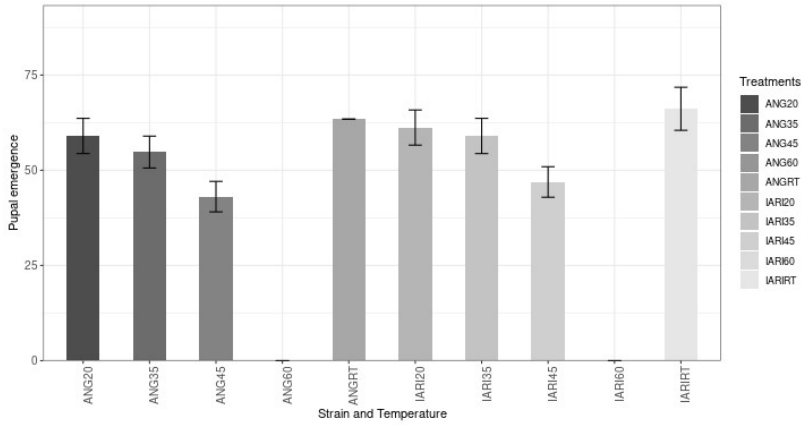


Figure 4. Effect of temperature on pupal emergence in Angamaly and IARI strain

IARI and Angamaly strains respectively. No larvae were recovered after treatment at 60°C (Figure 3).

The influence of temperature on pupal emergence when studied also showed similar results in this study. The pupal emergence was highest at room temperature for both Angamaly and IARI strains. Then the percentage emergence reduced with an increase in temperature. The least emergence was found in Angamaly strain at 45°C. No adult emergence was observed after heat treatment at 60°C (Figure 4).

The ability of an insect to tolerate temperature stress was reported to be varying corresponding to the stage of its development (Davison *et al*., 1969; Bursel *et al.,* 1973). In this study, the adults were found to be more tolerant to heat followed by the larval stage and the pupal stage was found to be most affected by temperature stress. Soderstrom *et al.* (1992) reported high mortality among adults when exposed to temperatures more than 38°C at 24 and 48h of incubation. In certain studies, the younger *T. castaneum* larvae were reported to be the most tolerant stage due to higher metabolic activity and *hsp* production (Mahroof *et al.,* 2003a).

The effect of temperature on different insects has been studied for exploiting the use of a controlled environment as an effective pest control method. The use of fluctuating temperatures was one such suggested method, however they were found to be increasing lifespan in some insects like *Trichogramma* (Consoli and Parra 1995; Easterling *et al.,* 2000), decreasing the lifespan in insects like *Cotesia marginiventris* (Butler and Trumble, 2010) and even reports of not showing any significant changes in lifespan of *Scatophaga stercoraria* (Kjærsgaard *et al.,* 2013).

Fluctuating temperatures were also reported to be affecting the morphology of certain insects, *ie.,* the pupal size, thorax size, wing size and venation, body size and weight of insects affected when exposed to fluctuating temperatures (Petavy *et al.,* 2004; Czarnoleski *et al.,* 2013). In the case of honeybees, their behavior and physical structures were found to be influenced by temperature exposure during their pupal stage (Tautz *et al*., 2003).

Zhang *et al.* (2015) in their study on chlorpyrifos-resistant DBM found that the pupal survival rate and adult emergence, when exposed to higher temperatures were higher in susceptible strains than that of resistant DBM. The wing venation was also severely affected in case of resistant adults compared to that of susceptible insects.

Bhugio and Wilkins (2021) investigated certain non-defensive attributes that included biochemical and physiological properties, which may be attributed to fitness cost to the insecticide resistance developed in RFB. They found that there was a significant reduction in the amount of food consumed by a resistant strain of the insect. They also showed that the width and weight of the gut tract of the resistant RFB were also lower compared to the susceptible counterparts. The study also indicated that insecticide resistance had influenced the metamorphosis in resistant strain of the test insect.

The present study showed that temperature exposure for a period of 1h did not significantly affect the adults of the test insect. However, the survival of the larvae and the pupal emergence was affected by the treatment. In all the stages, the susceptible insects had better survival than the resistant insect.

5.3. Expression analysis of *Tchsp70* gene in malathion resistant and susceptible strains of *T. castaneum* after heat treatment

One of the most important responses to thermal stress by any organism is the production of heat shock proteins and this has been documented in many species. The *hsps* are reported to have a significant role in the development and are documented in different species of insects (Huang *et al.,* 2009; Jiang *et al.,* 2012). There have been reports about the upregulation of *hsp* gene expression as a response to thermal stress in many insect species and a few reports of them not being related to conferring thermotolerance (Zhao *et al.,* 2012). The induction of *hsps* is reported to be rapid and transient (Huang *et al.,* 2007; Xu *et al.,* 2014; Cheng *et al*., 2016).

Tribolium, like other insects, is also found to be triggering *hsp* genes upon exposure to different stresses that includes temperature. Several heat-induced genes were reported in *T. castaneum*, which include *hsp21.6, hsp60, hsp70, hsc70* and *hsp83,* which is a homolog to *hsp90* (Xu *et al*., 2010).

In this study, the relative expression of *Tchsp70* at different temperatures provided insight into the involvement of *Tchsp70* gene expression to thermal stress in different stages of the insect in both malathion resistant (Angamaly) and susceptible (IARI) strains.

In case of adults, the *Tchsp70* expression in both resistant and susceptible strains was found to be increasing with an increase in temperature from 20℃ to 60℃. However, there was no significant increase in the fold expression in susceptible adults as that of the

resistant adults, when compared to the control insect (IARI strain) which was taken without any temperature treatment (Figure 5). The IARI strain showed a 110.38-fold expression when exposed to 20℃ compared to the control insect, and the expression increased to 197.23 times at 35℃, 208.17 times at 45℃ and 260.01 times when exposed to 60℃. While in Angamaly adults the fold expression was found to be higher compared to that of the susceptible strain. From a 999- fold expression at 20℃, the expression increased 14523 times (35℃), 35769 times (45℃) and then reduced to 16615 times when exposed to 60℃ for 1h.

Similarly, the larvae of Angamaly and IARI strains showed an increase in *Tchsp70* expression from 20℃ to 45℃ and then the expression reduced when the temperature was further increased to 60℃. Both the strains showed similar pattern however, the expression in the susceptible strain was found to be higher than that of the resistant strain at 20℃, 35℃ and 60℃. An exception was found at 45℃, where the expression in resistant strain was found to be higher than susceptible strain (Figure 6).

The expression of *Tchsp70* in Angamaly and IARI pupae was also found to be increasing with the increase in temperature until 45℃ and then decrease when the temperature was increased to 60℃. The *Tchsp70* expression in resistant pupa was found to be higher than that of susceptible at all temperatures except at 20℃ where the expression in susceptible was higher than that of the resistant strain (Figure 7).

The present study clearly indicates that the relative expression of *Tchsp70* in Angamaly strains when compared to that of the susceptible strain showed an overall increase as the temperature was increased. The expression was observed to be the highest in adults, followed by the pupae and then larvae (Figure 8). The peak expression in each of the developmental stages were observed at 45℃, indicating that there is an upregulation in *Tchsp70* in RFB when thermal stress was introduced.

Similar reports were reported in *T. castaneum,* where the *Tchsp70* and *Tchsp90* expression was found to be reaching the maximum in adult stages compared to other stages of the insect. A study on *Macrocentrus cingulum* showed that *hsp70* and *hsp90* were highly expressed when exposed to stress (Xu *et al.,* 2010). Zou *et al.,* (2012) however reported that the expression of *hsp90* under stress conditions was higher than that of *hsp70* in *Thitarodes pui*.

Similar reports of thermal-induced *hsp* are well documented in almost all organisms. Mahroof *et al.* (2005b) studied the expression of three different tc*hsp70* genes (*tchsp70I, tchsp70II* and *tchsp70III*) in every life stage of *T. castaneum* when exposed to temperature stress and reported that the gene is stress inducible in all the life stages. The study showed that *tchsp70I* and *tchsp70III* were upregulated in young larvae and adults and downregulated in old larvae and pupae indicating the genes involved in stress response and developmental regulation. However, *tchsp70II* showed insignificant changes at elevated temperatures.

Zhang *et al.* (2015) studied the expression pattern of *hsp70* and *hsp90* in chlorpyrifos resistant and susceptible DBM pupae and adults when exposed to temperature and reported that the basal expression in susceptible pupae was lower, however upon temperature exposure, there were instances of higher upregulated expression of the genes than that of the resistant strain. In adults, the susceptible adults showed similar or lower expression than that of resistant adults. They also mentioned that the expression of the genes increased with increasing temperature until it reached 47°C and then showed a reduced expression at 50°C.

Yi *et al.* (2018) studied the expression of six *hsp* genes in *Trichogramma chilonis* and reported that the expression of *Tchsp70* and *Tchsp90* was highest in the adult stages compared to the larval stage during its development. Upon exposure to different temperatures, there was upregulation of the genes in all the life stages and the expression was greatly upregulated at temperatures 32 to 40°C. The result also suggested that no significant changes were observed in expression at colder temperatures. They also reported that the expression of *Tchsps* at 40°C was the maximum observed. The expression of all the genes was dependent on the exposure time *ie.,* there was an increase in expression for all samples exposed beyond 1h, except for *Tchsp21.6*, which showed reduced expression with subsequent incubation time.

5.4. Evaluation of susceptibility of malathion resistant strain of *T. castaneum* when exposed to heat treatment.

Malathion was one of the recommended and most widely used insecticides at storage facilities. Although its use has now been banned in India, there are still reports of malathion resistance in RFB collected from different FCI godowns. In this study too, the

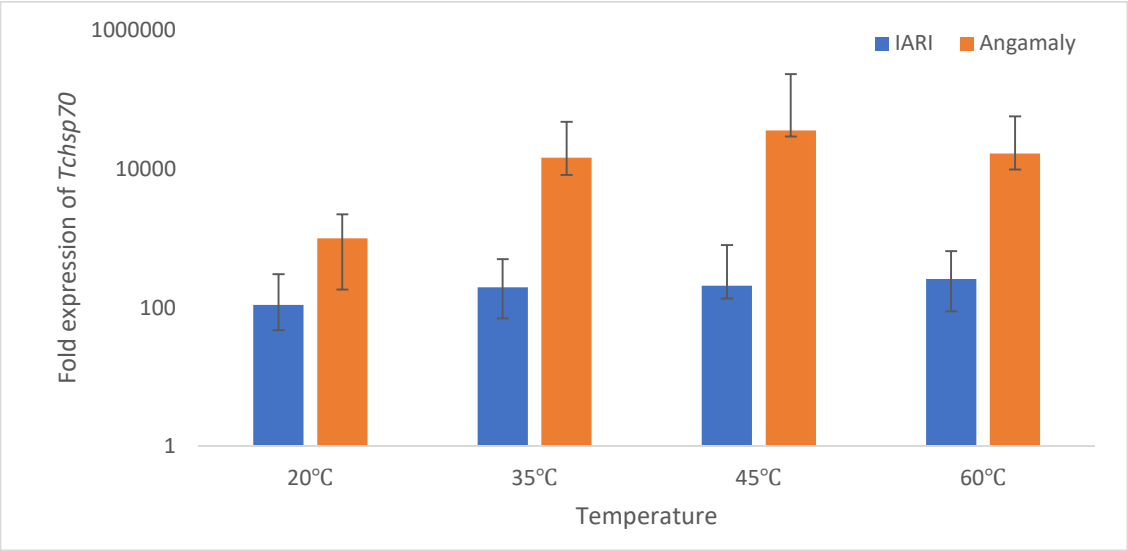


Figure 5. Relative *Tchsp70* expression in IARI (susceptible) and Angamaly (resistant) *T. castaneum* adults taking IARI adults without temperature treatment as control

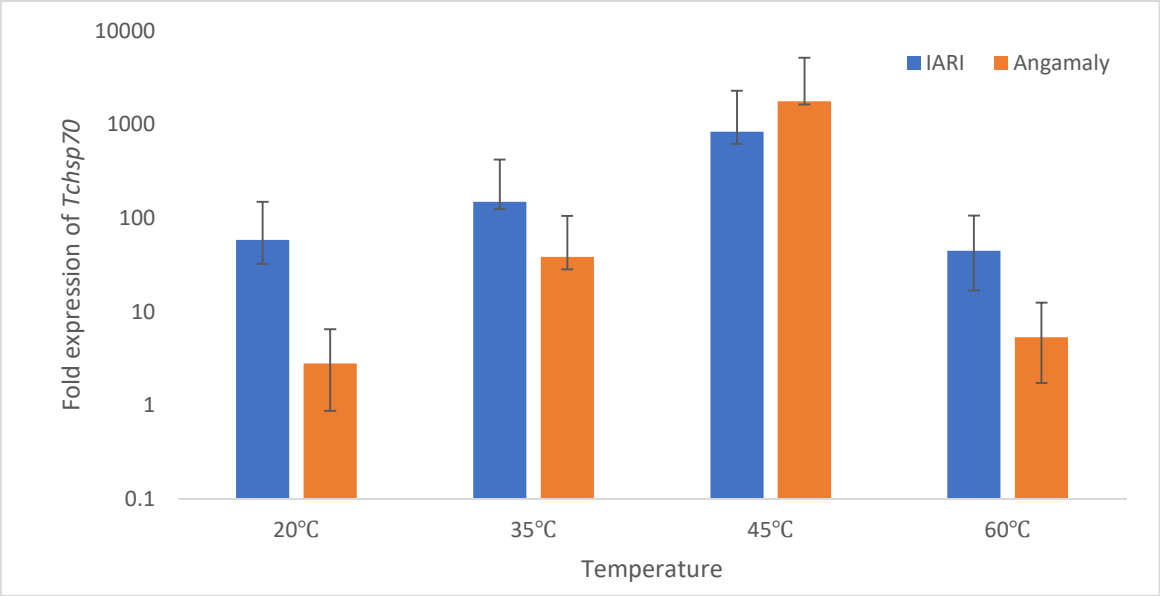


Figure 6. Relative *Tchsp70* expression in IARI (susceptible) and Angamaly (resistant) *T. castaneum* larva taking IARI larvae without temperature treatment as control

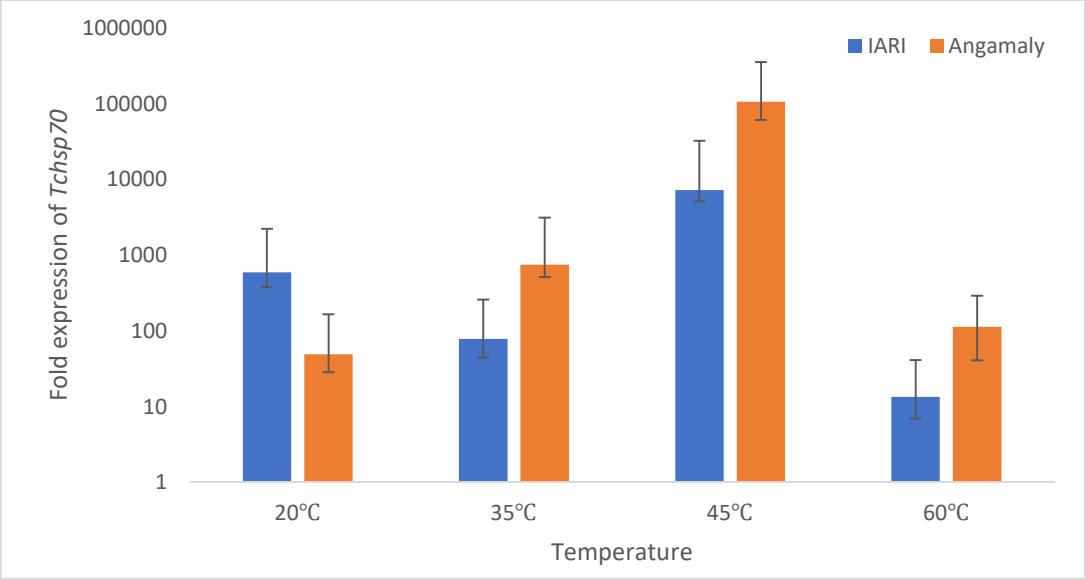


Figure 7. Relative *Tchsp70* expression in Angamaly (resistant) and IARI (susceptible) strains of *T. castaneum* pupa taking IARI adults without temperature treatment as control

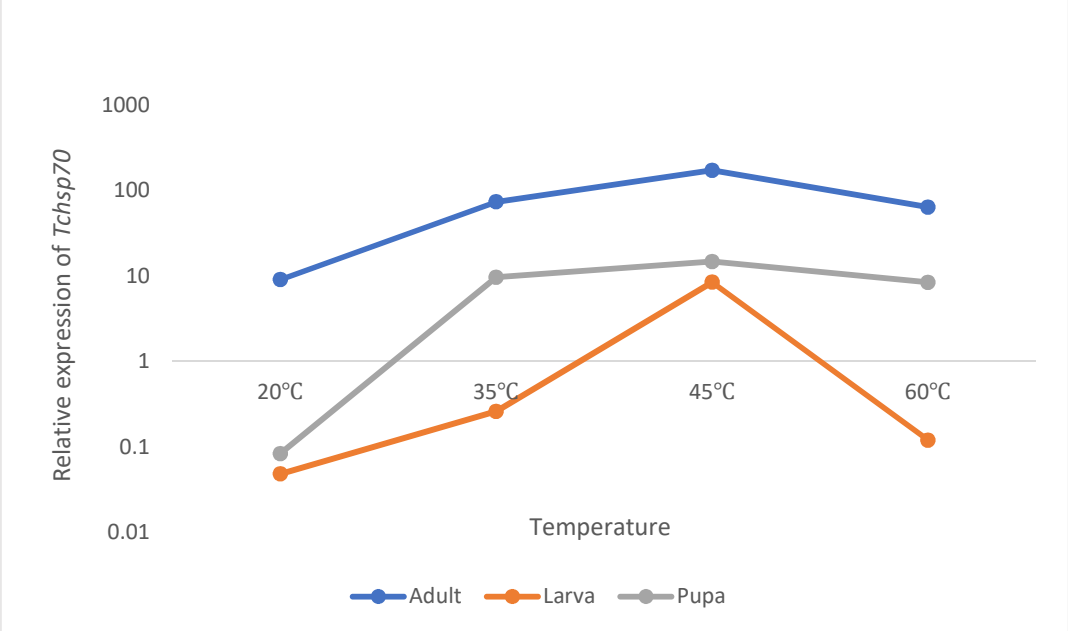


Figure 8. Relative *TcHsp70* expression in different life stages of malathion resistant *T. castaneum* at different temperatures

strain collected from FCI, Angamaly showed resistance to malathion when studied using residual film bioassay.

In Angamaly strain, LC_{50} value was found to be maximum at room temperature (25.8-26℃; 81% R.H.) which was 3020.0 ppm with a resistance ratio of 16.78 when compared to that of susceptible strain. When exposed to temperature, the LC_{50} values showed significant changes in both Angamaly and IARI populations. The LC_{50} values for the resistant strain were found to be decreasing with the temperature exposure *i.e.,* 700.0 ppm at 20℃ which then increased to 900.0 at 35℃ and then again showing a downfall to 700.0 at 45℃. This indicates that the sudden temperature exposure influenced malathion resistance on the resistant strain.

However, in the susceptible strain there was little difference in LC_{50} value. The study showed that the LC_{50} value increased as the temperature was increased *ie.,* 100.0ppm at 20℃, 150.0ppm at 35℃ and the LC_{50} peaked for the susceptible strain at 45℃ with a value of 240.0ppm. It was found that for the susceptible insects, the resistance increased slightly when the temperature was increased and for the resistant strains the resistance reduced as the temperature was increased.

Considering the resistance ratio, there was a significant reduction in the resistance ratio when compared to the susceptible strain. From 16.78 at room temperature the value dropped to 7.00 (20℃), 6.20(35℃) and the least value observed was 2.92 at 45℃. This clearly indicated that the susceptibility of the resistant strain of the insects was affected when exposed to temperature stress (Table 16).

The LC_{50} value observed in this study was lower compared to the previous studies (Anusree *et al*., 2019). The report of malathion resistance in *T. castaneum* dates back to 1962 (Parkin *et al.,* 1962) and in India, the first documentation of RFB malathion resistance was done by Bhatia *et al.* (1971). Since then, the resistance status of the insect was widely studied to have a better understanding of the resistance mechanism involved and to improve the management efficiency to control storage pests at a check. The resistance of the insects reported by various studies conducted at different parts of the country ranged from 16-37.76-fold (Rajak *et al.,* 1973; Pasalu and Bhatia, 1983; Srivastava *et al*., 2001). Currently, almost all the FCI godowns of the country are affected by the *T. castaneum* and found to be completely replacing the susceptible phenotypes. These natural populations collected from different storage sites were also found to be

displaying stable resistance gene frequency even after withdrawing the pesticide from their environment (Arnaud and Haubruge, 2002).

Table 16. Resistance ratio of Angamaly strain of *Tribolium castaneum* to malathion after heat treatment

Temperature (°C)	Resistance ratio	
	LC_{50}	LC_{90}
20	7.00	4.63
35	6.20	4.77
45	2.92	1.91
RT*	16.78	27.15

RT*: Room temperature (25.8-26°C; 81% R.H.)

Anusree *et al.* (2019) reported for the first time, the resistance status of *T. castaneum* to different pesticides used in FCI godowns in Kerala. The study was conducted on five FCI godowns in Kerala, and reported malathion, deltamethrin and dichlorvos. The study showed that the Angamaly strain had the maximum LC_{50} value of 6949.80ppm with a resistance ratio of 13.34. The study also suggested that the resistance developed uniformly in all the strains can be virtue of the common protocol being followed across the godowns.

The present study suggests that there is a significant tradeoff between thermal resistance and insecticide resistance of the test insect. The increase in susceptibility of *P. xylostella* towards deltamethrin to increasing temperature suggested the increase in toxicity in the insect. The study also suggested that the use of *Bt Cry1 Ac* along with deltamethrin can provide an effective control against the insect (Jaleel *et al.* ,2020). Toth and Sparks (1990) reported that the correlation between temperature coefficient and pyrethroids can be positive or negative depending on the species of the insect. In our study the results are similar, where the increase in temperature has increased the toxicity of malathion to resistant strain, however there is no significant change in the susceptible strain indicating that the increase in metabolic activity to counter the thermal stress in resistant strain had a cost on the fitness of the insect.

5.5. Fitness cost in malathion resistant *T. castaneum* when exposed to temperature stress.

Insecticide resistance and its associated fitness costs on the biology of the insects have been reported in many insects like *Nilaparvata lugens* (Puinean *et al.,* 2010), *A. aegypti* (Martins *et al.,* 2012; Alvarez-Gonzalez *et al.,* 2017) and *M. domestica* (Abbas *et al.,* 2017). In all these resistant insects, significant disadvantages or high energy costs have been reported when compared to their susceptible counterparts, even with the same genetic background (Carrière *et al.,* 2004; Gassmann *et al.,* 2009).

In this study, considering the biology of both malathion-resistant and susceptible insects, the life cycle of the resistant strain was found to be longer than that of its susceptible counterpart. Suggesting that there is a significant life trait related to fitness associated with the resistance developed. Changes in mortality of the insect, eggs per female, change in the developmental period, hatching percentage, and changes in pupal weight associated with insecticide resistance were reported earlier (Groeters *et al.,* 1994). Delnat *et al.* (2019) demonstrated that in certain mosquito species, there was an increased vulnerability to a biopesticide as a result of insecticide resistance developed.

Temperature is a critical abiotic factor for the survival, reproduction and distribution of any organism. In this study, the survival of adult, larvae and pupal emergence of both the strains also showed differences when exposed to different temperatures. The susceptible strain was found to be exhibiting a higher fitness compared to their resistant counterparts when exposed to different temperatures. This change in survival of the life stages can be attributed to the fact that the synthesis of *hsps* in many organisms is proven to create stresses on different metabolic activities due to the excess energy requirement arising simultaneously (Krebs *et al.,* 1998).

Also, over-expression of *hsp70* were also reported to be detrimental, exhibiting fitness cost in many organisms. In this study, there were evidence of upregulated expression of Tc*hsp70* in malathion resistant *T. castaneum* adults as the temperature was increased from 20 to 45°C, and correspondingly a significant fitness cost was observed in its resistance towards malathion. The LC_{50} was found to be lower at 20 and 45°C, suggesting the production of *Tchsp70* has increased the susceptibility of the adults to the chemical. The bioassay also suggested that the susceptibility of the susceptible strain did not differ significantly with different temperatures,

Similar records were found in a study conducted on chlorpyrifos-resistant DBM, the susceptible strains were found to display higher physiological and biological fitness as compared to their resistant counterparts when exposed to higher temperature stress. The wing venations were found to be damaged, and this was attributed to the temperature stress response of the pupa. The susceptible adults that emerged from heat-treated pupae were found to be having lesser damage to that of the resistant adults indicating fitness cost in the insects (Zhang *et al.,* 2015). In *Laodelphax striella*, when studied under 24 and 30℃, the fertility was higher in chlorpyrifos-susceptible than its resistant counterpart (Wang *et al.,* 2017). Similarly, *N. lugens,* when exposed to manipulated temperatures, showed that chlorpyrifos-resistant insects showed lower fecundity at most of the temperature treatments studied except at 18℃ and showed a longer recovery period from the stress (Yang *et al.,* 2018). Insecticide resistance developed by the insect is indeed a challenge and an area with tremendous practical application in controlling pests. The fitness costs associated with insecticide resistance developed can be used to provide a more effective and sustainable pest control method.

SUMMARY

6. SUMMARY

The study entitled "Temperature induced changes in the biology and heat shock protein gene expression" in red flour beetle, *Tribolium castaneum,* Herbst. (Tenebrioniidae: Coleoptera)" was carried out in the laboratories of Centre for Plant Biotechnology and Molecular Biology, Pesticides Residue Testing Laboratory, College of Agriculture, and Central Instrumentation Laboratory, Kerala Veterinary and Animal Science University, Thrissur dring 2019-2022.

The study comprised the evaluation of the effect of temperature on the survival of larval, pupal, and adult stages of *T. castaneum*, documentation of the relative expression of *Tchsp70* in different stages of the insect when subjected to temperature stress, and investigation of temperature induced changes in the susceptibility towards malathion in both malathion resistant and susceptible strains. The salient features of the study are summarized below.

- Two strains, malathion resistant strain, collected from FCI, Angamaly and susceptible strain from Division of Entomology IARI were reared and sub-cultured during the study.
- The duration of different life stages *viz.,* larval, pupal, and adult stages of malathion resistant strain was found to be slightly longer than the susceptible strain.
- The life stages were exposed to 20℃, 35℃, 45℃ and 60℃ for 1h. None of the life stages insect were recovered after the exposure at 60℃.
- The temperature treatments (20℃, 35℃ and 45℃) did not affect the mortality of the adult stages of both resistant and susceptible strain. However, the larval mortality and pupal emergence were affected with the increase in temperature.
- The resistant strain was found to be vulnerable to the sudden temperature stress compared to the susceptible.
- Total RNA was isolated from larval, pupal, and adult stages of both malathion resistant and susceptible strain after temperature treatment and recovery period.
- A pair of primer for the *Tchsp70* gene was used for gene expression analysis.
- The *Tchsp70* gene was found to be heat inducible. The expression was found to be increasing with the temperature exposed and peaked at 45℃.

- The gene expression was highest for the adult stages followed by the pupal and larval stage. The resistant strain, in most cases were found to show higher expression compared to the susceptible.
- The peak expression was recorded for adult stage of the resistant strain when exposed at 45℃.
- The malathion resistance in the resistance strain was confirmed using residual film bioassay.
- The LC_{50} values showed that the resistant strain was found to be susceptible to lower concentrations following the temperature treatment at 20℃ and 45℃.
- The susceptible strain however did not show any significant changes in the LC_{50} values among the temperature treatments.
- The resistance ratio was recorded to be more than 20 at room temperature, however the ratio dropped to 4 at 20℃ and 35℃. The resistance ratio was 1.91 when the temperature exposed was 45℃.
- Thus, suggesting that there is a possibility of fitness cost associated with the resistance developed that can be exploited for better control of this insect.

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TEMPERATURE INDUCED CHANGES IN THE BIOLOGY AND HEAT SHOCK PROTEIN GENE EXPRESSION IN MALATHION RESISTANT RED FLOUR BEETLE, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

by

SANJAY SABU
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ABSTRACT OF THE THESIS

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ABSTRACT

Post-harvest loss in weight and calorific content is a major threat to meet the dietary needs of ever-growing global population. Among the factors responsible for post-harvest losses in India, insect pests most prominently red flour beetle (RFB), *Tribolium castaneum* (Herbst) are reported to be responsible for maximum loss in storage godowns and silos. Both adult and larval stages of this insect can cause damage to stored products and deteriorate the quality. Different methods are employed to control this pest in storage places. Using pesticides has been an effective, economical, and fast method to keep the insect population at check. In Food Corporation of India (FCI) godowns, malathion along with dichlorvos and deltamethrin were recommended for controlling the storage pests. However, *T. castaneum* are known for developing resistance against different pesticides and the resistance was reported as early as 1971. A study on the resistance status of malathion in RFB from different FCI godowns in Kerala reported the strains collected from FCI, Angamaly showed the maximum resistance (LC_{50} value of 6949.80 ppm).

Although insecticide resistance is beneficial for the insects, they are often achieved by regulatory decisions which can cause developmental or biological consequences in the insect. Many insects have been reported to exhibit fitness costs associated with resistance developed. This study was proposed to study if any fitness cost is associated with malathion resistance in RFB, understanding which can enable us to opt for a safer and effective control strategy.

To study the effect of malathion resistance, two strains were taken. Malathion resistant strain from FCI godown, Angamali, and malathion susceptible, IARI strain, which was maintained without pesticide exposure at Pesticides Residue Laboratory, College of Agriculture. Comparing the life cycle of both Angamali and IARI strains, the Angamali strain showed longer developmental period during larval stage compared to the susceptible strain. The egg, larval and pupal periods of Angamali and IARI strains were observed to be 4.6 ± 0.52 and 3.4 ± 0.52, 26.3 ± 0.67 and 24.6 ± 0.84, and 5.2 ± 0.79 and 4.4 ± 0.7 respectively. Longer developmental period was reported in many insects as a penalty to the insecticide resistance developed.

The effect of temperature on the mortality of different life stages of RFB were studied and the adults of both Angamali and IARI strains were found to be unaffected.

However, the larval and pupal stages were found to be affected. Pupal emergence was found to be highest at room temperature i.e., 83.3% for Angamali and 80.0% for IARI, which was then found to be affected as the temperature was altered. Angamaly strain showed maximum penalty when compared to IARI.

The heat shock proteins (hsps) are mostly upregulated when the organism is under any stress to protects its cellular components and other proteins. However, the over expression of *hsps* is found to be expensive for the organisms. In our study, the *Tchsp70* gene was found to be thermal induced, and the relative expression peaked in adults at 45℃, nearly 171 times than that of the susceptible adults, followed by the pupal and larval stages. This study showed that *Tchsp70* in RFB is heat inducible. The over expression of *hsp70* were also reported to be detrimental, exhibiting fitness cost in many organisms and even creating stress on different metabolic activities due to the excess energy requirement.

Evaluation of susceptibility to malathion when the resistant strains where subjected to temperature stress was studied and the results showed that there was significant reduction in the LC_{50} value of the resistant strain. At room temperature the LC_{50} value for Angamali strain was found to be as high as 3020.0 ppm, however the value dropped at 20℃, 30℃ and 45 ℃ to 700.0ppm, 900.0ppm and 700.0ppm respectively. This suggests that the expenditure for countering the heat stress i.e., for producing *Tchsp70* have given the resistant insect a penalty and thereby increasing its susceptibility to lower doses of malathion. On the contrary, the susceptible strains exposed to temperature were found to have gained slight resistance to malathion. The LC_{50} value for susceptible at 20℃ was 100.0ppm, which rose to 150ppm at 35 ℃, and then to 240ppm at 45℃. This showed that the over expression of *Tchsp70* in susceptible strain was found to benefit the insect and then give penalty for the resistant insect, suggesting that there is considerable fitness cost associated with malathion resistance in *T. castaneum,* and potential use of temperature or controlling environment for storage can reduce the dependence on higher dose of insecticide.

Insecticide resistance developed by the insect is indeed a challenge and an area with tremendous practical application in controlling pests. The fitness costs associated with insecticide resistance developed can be used to provide a more effective and sustainable pest control method.