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Molecular basis of growth and aging in model organisms with special reference to the silkworm *Bombyx mori*: A review

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Abstract
An attempt was made to understand the basis of growth and ageing through the works on few model organisms and validate silkworm as an appropriate insect model for the study of growth and longevity. The first part of the review embodies research work in the mammalian and sub-mammalian systems whereas the second part presents the literature survey using insects and *Bombyx mori* in particular for ageing studies.

Keywords: Growth, ageing, longevity etc.

Introduction
Sericulture is an agro-based, labour-intensive, cottage industry for the production of raw silk and its byproducts. The history of sericulture dates back to 2500 BC when the practice of silkworm rearing started in China (Kuhn, 1988) [56]. The art of silkworm rearing practice spread from China to rest of the world leading to the establishment of sericulture industry in many countries, including India. The importance of sericulture was realized in 1950's when it contributed to the economic growth of India and became a prominent cash crop. Today, sericulture using mulberry and non-mulberry silkworms is practiced by a large number of farmers in India. Additionally, with the recent advances in biotechnology and biomedical research, the silk-producing organism is gaining more importance as a laboratory model till date.

The domesticated mulberry silkworm, *Bombyx mori* has a long history as an organism of economic importance. The earliest known silk textile is about 5000 years old (Kuhn, 1988) [56] which bears out well with the observation that the domesticated silkworm, *B. mori* originated from its wild relative, *Bombyx mandarina*. The silkworm is a holometabolous insect belonging to the order Lepidoptera. The range of food selection of this insect is very narrow and limited to only mulberry leaves (*Morus* species) and hence the silkworm is classified as a monophagous insect. The life cycle of the silkworm is about 50 days with four distinct stages-egg, larva, pupa and adult (moth). The larval stage is the only stage during which food is ingested. The egg, larval and pupal stages are the growth stages and the moth is the sexually mature stage which lays eggs and later reaches senescence. Among the growth stages, the larval stage is apparently most significant as it undergoes ten thousand fold increases in weight and about seven thousand fold increases in size and spins cocoons by the end of its weight gain. Also the larval stage is characterized by intermittent moults (usually four and hence, the name 'tetramoulter'). The adult stage begins with the emergence of moths from cocoons. The duration of adult lifespan differs among various silkworm strains. The mean adult lifespan of *B. mori* is around 7-10 days for females and 4-5 days for males [136]. Although the adult stage apparently holds no importance in the sericulture industry from economic point of view, a study has been conducted to correlate adult life span with economic traits in silkworm (Anantha, 2010) [3].

Biogerontology, the study of the biological basis of ageing, has so far unveiled few preliminary concepts of ageing by describing age-related changes in organisms, organs, tissue cells and macromolecules (Holiday, 1995; Rattan, 2004) [35, 100]. Over the past 30 years, a great deal has been learnt about some of the long-standing mysteries of life such as development and behavior. Yet, the exact mechanisms underlying how we age, a topic of personal and scientific interest for more than two millennia, remain largely unknown.

Given the current demographic trends and the associated health care costs of our ageing societies, it is hard to imagine a more compelling area of biomedical research than basic research on the biology and pathobiology of ageing (Rattan, 2012) [99]. Intrinsic biological ageing is the major risk factor for virtually all of the major diseases of the developed societies. Although the optimal treatment of each and every disease, irrespective of age, is a social and moral necessity, preventing the onset of age-related diseases by intervening in the basic process of aging is the best solution for improving the quality of human life in old age (Farrelly, 2010, Rattan, 2005) [20, 98]. In comparison with research on mutagens, carcinogens and teratogens, there is very little research on 'gerontogens'-environmental agents with the potential to accelerate the ages of onset or rates of development of ageing and associated components of senescence phenotype (Martin, 1987) [74]. Biogerontologists are currently focusing on drawing evidence from the genome sequences of closely related organisms that show considerable differences in their lifespan (Buffenstein *et al.*, 2011) [12]. This may give a valid clue to the age-specific gene expression signals and form a base for further exploration on this front towards understanding ageing.

Growth and Ageing: A biological continuum

As we grow, we age. It is commonly assumed that growth and ageing are somehow linked, but the nature of this link has been elusive. At first glance, growth and ageing appear to be opposites. Growth is energy-driven synthesis of macromolecules from simple nutrients, an increase of order and a decrease of entropy. Ageing is decay, a loss of order and an increase in entropy. Seemingly, growth and ageing are mutually exclusive. Forever proliferating cells such as hydra do not show signs of ageing. In contrast, when an organism ceases to grow, ageing follows. However, manipulations that decrease growth, also decrease ageing and prolong lifespan. Hence, the question- whether growth and ageing are mechanistically similar or not, arises. As we discuss here, growth and ageing may not be opposites, but rather a continuation of one another driven by the same biological pathway. Ageing and growth may be linked in a way that growth produces ageing. In other words, excessive growth is a driving force for ageing. The biological pathway that drives both growth and ageing appears to be evolutionary conserved (Blagosklonny & Hall, 2009) [10].

Concepts of Ageing

Ageing is a universal process that began with the origin of life about 3.5 billion years ago (Harman, 2001) [31]. Much of the confusion about ageing is wrapped up in attempts to define it. Most definitions of ageing emphasize a steady, progressive, irreversible decline in organismal performance (Helfand & Rogina, 2003) [32]. As we see it, ageing is a complex process that results in compromised biological functions of the organism and increased susceptibility to disease and death (Berdasco & Esteller, 2012) [9]. From the molecular point of view, ageing can be defined as a progressive shrinkage of the homeodynamic space and associated with stochastic (unpredicted) occurrence and progressive accumulation of molecular damage (Rattan, 2012) [99]. One approach to defining ageing is by examining how it is measured, i.e. longevity. 'Ageing' and 'longevity' are terms that are frequently used interchangeably, but it is important to make clear distinction between them. 'Ageing' is a process- a functional decline that is essentially a byproduct of natural selection operating to achieve the optimal balance of the available resources between the competing priorities of maintenance and reproduction. 'Longevity', in contrast, is a parameter- the length of time that individuals will remain alive in the absence of death from extrinsic causes, this being inversely proportional to the pace at which ageing occurs (Kirkwood, 2002) [50]. However, this method only provides information on survival and mortality rates and but fall short of helping understand the process of ageing.

Why do we age? This question has baffled scientists since a long time. There is substantial disagreement regarding even the basic nature of ageing. Over 50 years ago, Sir Peter Medawar's essay, "An unsolved problem in biology" (1952) placed the question of 'why we age?' into an evolutionary context (Medawar, 1957) [79]. He proposed that age measured relative to age of first reproductive capability was a factor in the evolution process. However, it has not provided answers to the mechanistic question of 'how does the process of ageing take place?' (Helfand & Rogina, 2003b) [33].

Theories of Ageing

Because ageing occurs for non-intuitive reasons and unfolds in complex ways, theory plays a pivotal role in its research. Biological, epidemiologic and demographic data have generated a number of theories that attempt to identify a cause or process to explain ageing, and its inevitable consequence, death. The theories formulated to explain ageing can be categorized into-evolutionary, molecular, cellular and systemic.

Evolutionary theories

Evolutionary theories argue that aging results from a decline in the force of natural selection. The mutation accumulation theory of ageing suggests that detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence (Medawar, 1952) [78]. The Disposable Soma Theory of Ageing argues that the somatic organism is effectively maintained only for reproductive success; afterward it is disposable. This theory gives the idea that longevity has a cost; the balance of resources invested in longevity versus reproductive fitness determines the lifespan. However, both the theories fail to provide a specific cause of ageing. The Theory of Antagonistic Pleiotropy suggests that some genes may be selected for beneficial effects early in life and yet have unselected deleterious effects with age, thereby contributing directly to ageing (Weinert & Timiras, 2003) [125].

Molecular theories

The gene regulation theory of ageing proposes that senescence results from changes in gene expression (Kanungo, 1975) [47]. It is clear that many genes show changes in gene expression with age (Pletcher *et al.*, 2002; Zou *et al.*, 2000) [97, 127] but not whether selection could act on genes that promote senescence directly (Kirkwood, 2002) [50].

Cellular Theories

The Free Radical Theory of Ageing was first proposed in 1957 (Harman, 1957) [29]. It states that free radical reactivity is inherent in biology and results in cumulative damage and senescence. Elevated levels of both oxidant-damaged DNA and protein are found in aged organisms (Beckman & Ames, 1998; Shringarpure & Davies, 2002) [8, 112]. Although, the

theory could be proved right in small, short-lived organisms (Larsen, 1993; Melov *et al.*, 2000) [61, 81] but it failed to hold good for larger, long-lived organisms such as mammals (Mehlhorn, 2003) [80].

Systemic Theories

Neuroendocrine theory (1954) proposes that ageing is due to changes in the neural and endocrine function that are crucial for: 1) co-ordinating communication and responsiveness of all body systems with the external environment, 2) Programming physiological responses to environmental stimuli and 3) maintaining an optimal functional state for reproduction and survival while responding to environmental demands. A modified version of the neuroendocrine theory, the 'Neuroendocrine-Immuno Theory' proposes that in the hierarchy of multi-system regulation throughout the sequential stages of life, there is a significant role for the interaction and integration of the neuroendocrine and immune systems.

However, in recent years, the search for a single cause of ageing, such as a single gene, or the decline of a key body system, has been replaced by the view of ageing as an extremely complex, multi-factorial process (Kowald & Kirkwood, 1996) [54]. Several processes may interact simultaneously and may operate at many levels of functional organization (Franceschi *et al.*, 2000) [25].

Genetic basis of Growth and Ageing

The genetics of growth and ageing has made substantial strides in the past decade. This progress has been confined primarily to model organisms, such as yeast, nematodes, fruit flies and mice in which some 35 genes that determine lifespan have been cloned. These genes encode a wide array of cellular functions, indicating that there must be multiple mechanisms of ageing. However, it is now clear that there are at least four broad physiological processes that play a role in ageing: metabolic control, resistance to stress, gene dysregulation, and genetic stability [41]. There is ample evidence from studies performed on yeast, fungi [41], nematodes (Johnson *et al.* 2000; Johnson, 2002) [43, 44], insects [104]; Tatar, 2001) [120], rodents and humans that mutations in certain genes can either prolong or shorten the lifespan, and cause premature ageing syndromes (Arking *et al.* 2002; Kuro-O *et al.* 1997; Yu *et al.* 1996; Martin & Oshima, 2000) [5, 57, 126, 75]. Life span has a remarkable range between species, approaching 1,000,000 fold across all phyla and 10-50 fold within groups of the same grade of organization (Carey & Judge, 2000; Finch, 1990; Finch & Austad, 2001) [13, 22, 23]. Recent developments on the genetics of ageing can be seen as several streams of effort. In general, humans show a relatively modest (<50%) heritability of lifespan (Herskind *et al.* 1996; Ljungquist *et al.* 1998) [34, 66]. Short-lived laboratory models (fruit flies, nematodes, mice, etc) are yielding rapid advances with the discovery of mutants that increase lifespan in association with altered mechanisms (Lin *et al.* 1998; Rogina *et al.* 2000; Taub *et al.* 1999) [65, 104, 119].

Although these early findings do not show that a conserved genetic program actually controls ageing process across animal phylogeny, it is striking how frequently findings of metabolic rate, insulin signaling and free radicals have emerged from very different approaches to ageing in nematodes and animals, for example (Apfeld & Kenyon, 1999; Kimura *et al.* 1997; Lin, 1998) [4, 48, 65]. These findings provide a hint that the genetic control of lifespan was already developed in the common ancestor of modern animals so that the subsequent evolution of lifespan was mediated by the quantitative changes in the control of metabolism through insulin and the production of free radicals (Finch & Ruvkun, 2001) [23]. Application of single gene mutant analysis in *Drosophila* has resulted in isolation of specific genes whose activity influences the rate of ageing (Parkes *et al.*, 1998 on SOD1 expression in motor neurons) [94]. Selective breeding studies demonstrated that ageing, a quantitative trait, was influenced by heritable genetic factors (Rose & Charlesworth, 1981; Rose, 1984; Luckinbll *et al.* 1984, Arking, 1987; [7, 105, 72, 6]. As these techniques are refined and applied on a greater number of genes, it seems likely that a comprehensive understanding of the genetic component of ageing will be within our grasp.

Molecular Basis of Growth and Ageing

Several avenues to study ageing have placed us on the threshold of understanding basic underlying mechanisms and one of them is the molecular basis of ageing. Different studies have demonstrated that molecular measures, such as, gene expression, can better describe events associated with ageing (63-Rodwell *et al.* 2004; Krishnamurthy, 2004; Zehner *et al.* 2009) [102, 55]. Candidate genes have been identified as molecular markers of ageing because their change in expression was more indicative of organism age than chronological age alone. There are several important molecular causes of ageing that come from current research. These are damaged by Reactive Oxygen Species (ROS) generated by metabolism, genome instability, genetically programmed extension mechanisms, cell death and systemic ageing (Johnson & Sinclair, 1999) [42].

Oxidative damage: The oxidative damage theory proposes that ROS which are generated by metabolism cause cumulative damage over a lifetime (Harman, 1981) [30]. A supporting evidence for the theory is provided by the transgenic *Drosophila* that over expresses both CU/Zn Superoxide dismutase and catalase and lives 34% longer than the controls (Orr & Sohal, 1994) [89]. Another possible genetic link between oxidative damage and ageing is provided by the long-lived 'age-1' mutant of *C. elegans* (Klass, 1983, Freidman & Johnson, 1988; Larsen *et al.*, 1995) [52, 26, 62].

Genome instability: The importance of specific kinds of genome instability in ageing is becoming increasingly apparent. The accumulation of genomic changes [i.e. point mutations (Szilard, 1959) [116], loss of repeated DNA sequences such as ribosomal DNA (Strehler, 1986) [114], rearrangements, and changes in chromosome number (Martin *et al.* 1985) [76] have been long proposed as the cause of ageing. In addition, telomere-shortening could behave as a molecular clock that signals the eventual growth arrest, termed 'replicative senescence' (Shay & Wright, 2007) [109]. Strong support for this notion include Telomerase Reverse Transcriptase (TERT) expression that maintains the telomere length can significantly delay ageing in mice (Tomas-Loba *et al.* 2008) [120] and this enzyme has also been identified in many organisms including silkworm, *B. mori* and flour beetle (Loo *et al.* 2000) [70].

Genetic programs: The universality of ageing phenotypes within a species argues for an underlying genetic program. The redistribution of the Sir complex from telomeres to

nucleolus in yeast is a specific molecular example of how a genetically programmed response can extend lifespan and also lead to a gradual change in phenotype.

Models Organisms for Growth and Ageing

Model organisms provide a key to understand the complex biological processes. Scientists have established a handful of model systems to understand and modulate the process of growth and ageing.

The major model systems used in ageing research are- Baker's yeast (*Saccharomyces cerevisiae*), Roundworm (*Caenorhabditis elegans*), Fruit fly (*Drosophila melanogaster*), Mouse (*Mus musculus*).

Baker's Yeast

Baker's yeast (*Saccharomyces cerevisiae)* is a microbial eukaryote that undergoes an asymmetric form of cell division called budding. The individual yeast cell buds a limited number of times, producing a daughter cell every time. The daughter cell has in principle, the capacity for a full replicative life span (Mortimer & Johnston, 1959; Muller *et al.* 1980) [84, 85]. The measure of the yeast life span is therefore the number of division of the mother cell before it dies and not chronological time (Mortimer & Johnston, 1959; Egilmez & Jazwinski, 1989; [84, 17]. Yeast has received a widespread acceptance as useful model in the recent years (Gershon & Gershon, 2000) [27]. Several features of the yeast predispose this organism to molecular studies of the aging process:

1. The cell and organism are one-this allows one to circumvent discussions of the relevance of cellular aging to the aging of the organism as a whole.
2. Intrinsic features of aging can be analyzed without confusion from the extra cellular factors.
3. The powerful tool of yeast genetics (Struhl, 1983) [115] can be applied to the studies of aging.
4. The basic phenomenon of aging has been described in yeast (Jazwinski *et al.* 1990) [40].
5. The individual aging cell can be isolated and followed.
6. Age-synchronized yeast cells can now be prepared in bulk quantities (Egilmez *et al.* 1990) [118].

Although yeast does not have an insulin-signaling pathway, they appear to have a precursor to that metabolic control pathway, the yeast adenylate cyclase and SCH9, which regulates stress resistance and longevity (Fabrizio *et al.* 2001) [19]. Studies of ageing in yeast have led to the conclusion that a gene involved in the silencing of chromatin may be a key regulator of ageing (Gottlieb & Esposito, 1989; Sinclair & Guarente, 1997) [28, 111]. An increase in rDNA silencing by Sir2 proteins seems to increase lifespan (Kaeberlein *et al.* 1999) [45]. The genetic analysis of ageing in yeast points to four broad physiological processes important for longevity. They are-metabolic control, resistance to stress, gene dysregulation and genetic stability. Finally, the RAS2 gene has emerged as a homeostatic device for longevity (Jazwinski, 1999) [41]. It is hypothesized that this gene also contributes to genetic stability. Stress resistance by RAS1, RAS2 & HSP104 genes and their effect of increased longevity was elucidated by Sharma *et al.* 1998 [108].

Roundworm

The nematode, *C. elegans* is a good model organism for ageing research since it has a relatively short lifespan and produces a large number of progeny. The first evidence for genetic regulation of lifespan came from studies with *C. elegans*. It was discovered that worms with mutations in the dauer formation (daf) genes, such as daf-2 and age-1 were able to bypass dauer formation and become long-lived adults (Larsen, 2001) [59]. Daf2 genes were shown to be homologous to mammalian genes encoding insulin receptor (IR) and IGF-1 receptor (Kimura *et al.* 1997) [48]. 'age-1' mutant showed higher levels of superoxide dismutase and catalase, which provides us a direct clue to the role of these enzymes in longevity (Larsen, 1993; Vanfleteren, 1993) [61, 121].

Many life extending mechanisms have been studied in *C. elegans* (Budovskaya *et al.* 2008) [11]. Out of them, the most studied one is the daf-16 transcription factor mechanism. It mediates longevity in response to reduced insulin/IGF-1 like signaling and regulates the expression of many age related genes (McElwee *et al.* 2003; Murphy *et al.* 2003) [77, 87], mainly down-regulating them and extending lifespan. Another mechanism is the reduced activity of the mitochondrial electron transport chain (ETC). Mutations in genes involved in the activity of mitochondrial ETC, such as isp-1 and clk-1, significantly increased lifespan (Feng & Hekimi, 2001; Lakowski, 1996) [21, 58]. Several transcription factors mediating dietary-restriction-induced longevity in *C. elegans* has been identified. Among them, SKN-1 is requires for the response to oxidative stress in adult worms (An & Blackwell, 2003; An *et al.* 2005) [1, 2]. It was recently found that two SKN-1 dependent genes, nlp-7 and cup-4 are specifically required for lifespan extension by dietary restriction (Park *et al.* 2010) [90].

C. elegans is the first multicellular organism whose whole genome has been fully sequenced, leading to the development of a DNA microarray covering the whole genome sequence of *C. elegans*. Global gene expression analysis using DNA microarray provides a transcriptional profile of ageing in *C. elegans*. Gene expression profiling has revealed that genes involved in dauer-regulation and insulin/IGF-1 signalling are upregulated during ageing (Lund *et al.* 2002) [73]. Expression of ins-17 & ins-18 (insulin homologs) is significantly increased and SIR2.1, the repressor of insulin signaling is decreased in aged worms. HSP16.2 is a stress-sensitive reporter that can predict longevity in *C. elegans* (Rea *et al.* 2005) [101]. Promoter-binding motif analysis revealed that elt-3/elt-5/elt-6 GATA transcription unit played a pivotal role in normal ageing of *C. elegans* (Budovskaya *et al.* 2008) [11]. The reproductive system too regulates ageing in *C. elegans*. Laser ablation of Z2 and Z3 germline precursors resulted in 60% longer lifespan than normal (Hsin & Kenyon, 1999) [36].

Fruitfly

The fruitfly, *Drosophila melanogaster* has been one of the most common genetic model systems employed to assess complex biological phenomena. The benefits of using *Drosophila* for studying ageing in particular include (a) its relative short lifespan (3 months) (b) easy maintenance, (c) environmental and genetic manipulations that alter lifespan (d) already available information on ageing, (e) availability of stocks containing altered genes (f) powerful molecular genetic techniques, (g) sequence of the full *Drosophila* genome (h) proven success in dissecting apart biological phenomena such as development (Helfand & Rogina, 2003b) [33]. In addition, the life history of *Drosophila* is divided into distinct morphological stages, so the period of growth and development and be readily distinguished from the sexually mature, adult phase. If ageing research is primarily involved

in trying to understand the changes that take place in cells and organs over time, then organisms such as *Drosophila*, which are almost entirely postmitotic (Arking, 1991) [7], are excellent model systems for such studies. *Drosophila* has been used as a model system for ageing research since 1916. Loeb and Northorp used *Drosophila* to show that lifespan obeyed the normal laws of chemistry and physics, similar to other bio processes (Loeb & Northorp, 1916; Loeb & Northorp, 1917) [67, 68]. In 1920s, Pearl and colleagues used *Drosophila* to show that lifespan is an inherited trait (Parker, 1921; Parker, 1922; Parker, 1923) [91, 92, 93]. In the 1950s, Smith and colleagues showed the importance of reproduction in determining lifespan and highlighting *Drosophila* as a model system for studying fitness trade-offs and lifespan (Smith, 1958; Smith, 1962) [112, 113]. Laboratory selection experiments in the 1980's have showed the plasticity of lifespan in *Drosophila* (Larsen & Clare, 1985; Rose, 1984) [71, 105]. In the 1990s, Yonemura *et al.* (1990) [135] revealed the mode of major gene inheritances in relationship to the lifespan in adult *Drosophila* and identified the gene concerned in longevity, designated as Jm (Jumyo gene). Manipulation experiments conducted on transgenic *Drosophila* showed extended lifespan (Tatar *et al.* 1997) [117]. It was earlier found that a decrease in ambient temperature to 18 ^{o}C cans double the lifespan in *Drosophila* (Miquel, 1976) [82]. Further, observations on the reproductive status led to conclude that mating and reproduction has negative correlation with longevity (Chapman *et al.* 1993; Chapman *et al.* 1995) [14, 15]. In the 2000s, it was shown that caloric or dietary restriction extended lifespan in a variety of organisms including *Drosophila* (Finch & Ruvkun, 2001; Koubova & Guarente, 2003; Longo & Finch, 2003) [23, 53, 69], but the molecular or physiological mechanisms by which life extension occurs is still in its preliminary stage.

Genetic approaches to understand ageing in *Drosophila* have revealed single gene alterations that extend lifespan including Indy, InR, chico and rpd^3 (Clancy *et al.* 2001; Rogina *et al.* 2002; Rogina, 2000; Tatar *et al.* 2001) [16, 103, 104, 120]. *Drosophila* has been studied extensively for testing the oxidative stress hypothesis. It was observed that decrease in enzyme, like catalase and superoxide dismutase (SOD) shorten lifespan, suggesting the importance of reactive oxygen species and lifespan (Kirby *et al.* 2002; Missirlis *et al.* 2001; Phillips *et al.* 1989; Phillips & Hilliker, 1990) [49, 95, 96]. Drugs like PBA (4-phenylbutyrate) fed to *Drosophila* have exhibited lifespan extension (Kang *et al.* 2002) [46].

Lifespan in *Drosophila* is a quantitative trait. QTL analysis of 98 RI strains of *Drosophila* revealed QTLs having moderate effect on longevity which were mapped to several genomic regions (Leips & Mackay, 2000, 2002; Vieira *et al.* 2000; Nuzhdin *et al.* 1997) [64, 127, 88]. Unfortunately, only a few genes affecting *Drosophila* lifespan have so far been isolated using QTL mapping. The only genes identified so far through this strategy are Ddc (De Luca *et al.* 2003) [129], stc & ms (2) 35ci, catsup (Carbone *et al.* 2006) [130], DoxA2 and tup (Mackay *et al.* 2006) [128].

Mouse (*Mus musculus*)

Mouse, the well-established laboratory model for multifarious research, is a chosen mammalian model for ageing research a well. In long-living mammals, a large number of factors affect the ageing process and the probability of death, including neoplasia, sepsis and organ-specific failure. Therefore, gene expression profiling of each organ is more appropriate for understanding of ageing. In mice, characterization of gene-expression patterns in different tissues using oligonucleotide DNA microarrays revealed that with ageing, genes involved in energy metabolism were decreased in expression, while the genes involved in stress-response (hs genes, anti-oxidant genes, etc.) were increased in expression, in gastrocnemius muscle. Comparison of the transcriptional profiles of the neocortex and cerebellum tissues of young and old mice revealed differential gene expression patterns.

In the stride to extend mammalian lifespan, scientists resorted to experiments with dietary restriction (DR) in mice. Transcriptional profiles from DR mice and normally fed mice showed that most transcriptional alterations during ageing are either completely or partially prevented by DR. Dietary supplementation with vitamin E showed partial preventive effect on age-related transcriptional changes in the aged heart in mice [10-47]. A mutation in the gene encoding the protein p66^{shc} (adaptor protein involved in the oxidative stress response) extends the lifespan of mice by about 30% [54-78]. It was found that the Ames dwarf mice, which have a defective PROP-1 gene, live approximately 50% longer than normal. [54-91]. To identify other gene candidates in the laboratory mouse genome, several groups of scientists are studying gene influences on lifespan in common mouse strains by analysis of quantitative trait loci (QTL). De Haan & Van Zant [31-18] have found a region on chromosome 11 with QTLs that influence lifespan.

Silkworm as a Lepidopteran model

Throughout the twentieth century, the Lepidopterans, especially moths have played important roles in fundamental studies in physiology, viz. end ocrinologic phenomena, and in biochemistry. Increasingly, they are also finding use in studies of biological development. Unfortunately the attention lavished on *D. melanogaster* as an insect species par excellence for the genetic and molecular analysis of development has eclipsed both past and current work on Lepidoptera (Goldsmith & Wilkins, 2005) [132]. Although studies with many Lepidoptera have made important contributions to genetics today, silkworm stands as the only member of this taxonomic group whose genetic system is well enough established to consider adopting it as a molecular genetic model for solving a broad range of fundamental biological problem.

The modern genetic analysis of silkworm goes back to the turn of the century and provided some of the first modern accounts of mutations. With respect to fundamental research today, work on silk moths has several major strands; the molecular biology of silk production whose analysis includes the special transcriptional and translational mechanisms employed by the silk gland; the study of the organization, evolution and regulation of the large complex chorion multi-gene family; the recently begun investigations of homeotic genes and mutant effects at the molecular level and the combined molecular and genetic analysis of embryogenesis (Goldsmith & Wilkins, 2005) [132]. The development of the genetics of the silkworm, *B. mori* continues to play a key part in all of this work.

In modern times, *Bombyx* has been used as a model for genetic studies since the birth of genetics as a formal science in the early 1900s. As early as 1905, Toyoma, one of the founders of silkworm genetics, initiated hybridization in silkworms. He first reported the discovery of chorion mutation that affects the shape and transparency of egg shell

in 1910 (Tazima, 1964) [131]. Japanese geneticists maintained active research into the fundamental principles of genetics keeping pace with the field and often pioneering with early reports of such phenomena as dominance, maternal inheritance, sex-linkage, homeotic mutants (Suzuki, 1929, Suzuki & Ohta, 1930-all cited by Tazima, 1964) [131], enzyme polymorphisms and unstable mutations (all cited by Goldsmith & Wilkins, 2005) [132].

Silkworm as a model to study growth and ageing

Silkworm is a holometabolous insect with four distinct stages in its lifecycle, viz., egg, larva, pupa and moth. The egg, larval and pupal stages are the growth stages and the moth is the sexually mature stage exhibiting aging. Thus, the mechanism of ageing in *Bombyx* is made up of at least two steps, the growth period, concerned in growth rate and the post growth period related to senescence rate (Akio Murakami, 1990a) [133]. Among the growth stages, the larval stage is morphometrically most significant as it undergoes ten thousand fold increase in size and about seven thousand fold increase in weight. Also the larval stage is characterized by intermittent molts (usually 4). However, the larval period is markedly not influenced by moltinism or the number of molts and the larval duration or the growth rate is under the influence of polymeric genes and environmental factors cause small variations (Akio Murakami, 1990a) [133]. In *Bombyx*, Murakami (1990b) [134] detected a sex-linked recessive gene-'pre' which accelerates growth rate in pupal stages.

Holometabolous insects undergo dramatic morphological changes during metamorphosis. In the larval stage, larval-specific tissues such as larval muscle, midgut, and salivary glands exhibit their function only during that stage and then undergo programmed cell death and histolysis during the prepupal phase of metamorphosis. To date, many mutation analysis has been carried out, in attempts to understand the fundamental processes that distinguished between the developmental stages in *B. mori* such as egg formation, embryonic patterning, larval epidermal pigmentation, wing disc development and diapauses, but the analysis of the entire gene set related to each of the developmental stage are limited. Studies of the gene expression profile for a specific developmental stage of *B. mori* has been reported through the use of high-density DNA microarray technology or SAGE. To identify genes that are important in silkworm development, gene expression profiling has been applied to embryonic and larval stages of *B. mori*. Among identified genes, RFeSP, the Rieske iron-sulfur protein, shows the ubiquitol-cytochrome-c oxidoreductase activity which is associated with lifespan.

Steroid hormones are responsible for the co-ordination and regulation of many aspects of development, growth and differentiation of multicellular organisms. In insects, ecdysteroids play a central role in development, especially in molting. In an attempt to understand the genetic basis of development, studied the dimoulting (mod) mutant *B. mori* strain that undergo metamorphosis early and complete the larval stage with two moults instead of the normal five. They identified the mod trait as a gene for a cytochrome P450 enzyme, CYP15C1 that was expressed in the corpora all ata, suggesting that factors other than juvenile hormone exists that prevents metamorphosis in the early larval instars. The mean adult lifespan of *B. mori* is around 7-10 days for females and 4-5 days for males (Akio Murakami, 1989) [86]. The duration of adult lifespan also differs among various silkworm strains, suggesting that the adult lifespan may be genetically controlled. Studies on adult lifespan of two different voltine groups of silkworm, *B. mori* revealed races belonging to the long-lived and short lived groups.

The adults of Daizo have the shortest known lifespan of 2 days. In a study using male and female moths of Daizo and J106 strains, it was found out that the lifespan specific to the strain and sex of adults depends on brain function in each strain and sex. It is suggested that adult lifespan of Daizo has a Mendelian monogenic hereditary mode and the responsible gene is located on a certain specific autosome. The short adult lifespan of Daizo strain is controlled by the single recessive 'sdi' gene (Murakami, 1989a) [86]. But the 'sdi' gene does not control adult lethality. Another strain from Japan, N251 has a short adult lifespan of 4 days and it is controlled by a single recessive gene 'sli'. In a survival analysis in Korea, a study on 277 silkworm varieties revealed that mating significantly affected adult lifespan, especially in males. (Ageing is a complex biological phenomenon. To find out the exact cause and the mechanism that drives an organism into senescence is the biggest challenge bio gerontologists face till today. Taking hints from other ageing models, a number of peripheral studies surrounding molecular pathways and factors that influence ageing, has been carried out in the silkworm, *B. mori*. Insulin-signalling pathway plays an important role in regulating growth, development, lifespan, as well as metabolic homeostasis. Insulin may specifically activate ecdysteroid in prothoracic gland. Insulin-like peptide, *Bombyx* in was identified in *B. mori* and it is encoded by BBX-B8 gene which has been shown to play an important role in organ development, reproduction and the meta bolization of trehalose in silkworm.

Autophagy plays an important role in various biological events including development, differentiation and determination of lifespan. A full set of genes and their encoded proteins of this evolutionary pathway have been identified in many eukaryotic animals from yeast to mammals. 2 out of 20 autophagy genes found in *B. mori* supposedly play an important role in maturation of silk glands.

Reactive Oxygen Species (ROS) such as hydrogen peroxide, superoxide anions, etc. accumulates in tissues and cells as a result of exposure to environmental pollutants. ROS is harmful to living organisms because they tend to give oxidative damage to proteins, nucleic acids and lipids. In this context ROS has been recognized to be related to ageing and lifespan. The role of antioxidants in preventing aging has been a rather controversial topic for bio gerontologists. Superoxide dismutase (SOD) is a metal loenzyme involved in defense reaction to ROS and thus considered to be involved in lifespan extension (Orr & Sohal, 1994; [89]. SOD have been identified and characterized in *B. mori*, but its role in longevity still remains unclear. Another antioxidant enzyme, catalase has been reported to be present ubiquitously in silkworm for scavenging ROS. The gene encoding catalase has been sequenced in *B. mori*. Calorie restriction is known to extend the lifespan in different species from yeast to mammals. Moderate calorie restriction was shown to extend lifespan in the silkworm by the downregulation of four proteins, namely, storage protein 1 (SP1), arylphorin (SP2), Imaginal Disc Growth Factor (IDGF) and 30 kDa protein. In this regard, Sirtuin2 (Sirt2) is a strong candidate to regulate calorie restriction and limiting calories resulted in activation of Sirt2. It is a kind of NAD+ dependent deacetylases ranging from bacteria to human and play an important role in many

biological processes mainly lifespan. Sirtuins link ageing, cancer and diet and thus are potential molecular targets for the development of pharmaceuticals to treat diseases. Sirt2 of *B. mori*, BmSirt2 was cloned and shown to be widely expressed in the silkworm body. In addition, the haemolymph of calorie-restricted silkworm larvae subjected to proteomic analysis revealed 19 differentially expressed proteins that may regulate lifespan of silkworm through calorie restriction. Dietary administration of 2, 2-Diphenylpropionate derivatives caused significant prolongation of the larval period. In another report using dietary supplementation, macro- and micro-nutrients were shown to yield positive effect on the adult longevity in the silkworm *B. mori*.

Heat shock proteins protect insects against heat stress. They have been shown to increase lifespan and resistance to oxidative stress in *Drosophila*. In *B. mori*, six hsp genes were reported. Protein expression analyses provide us a window to the basic understanding of fundamental processes of biology. Haemolymph proteins in silkworm have been subjected to myriad analysis, and to our concern, expression of genetic variation. A group of Korean scientists have analysed the haemolymph protein patterns of silkworm in terms of long and short life span through Native- and SDS-PAGE using 13 temperate races. The *B. mori* system with its excellent backup can be exploited to serve as an alternative model system to study gene expression [83]. Recent progress in areas of genetics, genome structure and functional genomics places silkworm in focus as a model lepidopteran for our entry into the genomic era [98].

Molecular Marker Systems with special reference to *Bombyx mori*:

The difference in the genetic makeup of individuals has led to the identification and development of molecular markers. A molecular marker is a gene or DNA sequence with a known location on a chromosome, or a protein sequence that can be used to identify individuals or species. With the advent of molecular markers, a new generation of markers has been introduced over the last two decades which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genetic and genomic analysis. Insects possess a vast undiscovered genetic diversity and gene pool that can be better explored using molecular marker techniques. Current trends of application of DNA marker techniques in diverse domains of insect studies show that mitochondrial DNA (mtDNA), microsatellites, Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSRs), Expressed Sequence Tags (ESTs) and Amplified Fragment Length Polymorphism (AFLP) markers have contributed significantly towards understanding genetic basis of insect diversity and for mapping economically and medically important genes and quantitative trait loci in insects. Besides, whole genome microarray and Single Nucleotide Polymorphisms (SNPs) assays are becoming more popular to screen genome-wide polymorphisms in fast and cost-effective manner [121].

With reference to mulberry silkworm, *B. mori*, more than 400 visible mutations have been placed in the linkage map which represent 217 loci consisting of mostly morphological and a few enzyme markers. The number of loci mapped so far is insufficient for a thorough understanding of the genome and for the analysis of the quantitative trait loci (QTLs) for important commercial characters in silkworm. Hence, the development of DNA based genetic markers in silkworm was initiated in the 1990s and preliminary linkage map using RFLPs and RAPD map of 169 loci were constructed. Later, a dense genetic map of silkworm covering all chromosomes based on 1018 RAPD markers was published. Later, an AFLP map of the silkworm was constructed with 356 markers. A genetic linkage map using 518 SSR markers was also established for *B. mori* [131]. Thereafter, to build a foundation for the complete genome analysis of the silkworm, *B. mori*, EST database was constructed covering about 55% of all the genes of silkworm. A linkage map for the silkworm *B. mori* was constructed based on SNPs [134]. The EST data of silkworm derived from the cDNA libraries has made it possible to mine SNPs from silkworm genome [137].

With regard to genome analysis in Indian silkworms, application of PCR-based markers, RAPD markers, and also DNA fingerprinting with minisatellite probes have been taken up to study the DNA profiling of silkworm genotypes. DNA profiling of silkworm has also been carried out using microsatellite markers, which has shown breed-specific profiles for 15 silkworms, clearly indicating the prospects of microsatellite markers for establishment of molecular Identities for distinguishing silkworm breeds. Genetic characterization by simple sequence repeats (SSR), inter-SSR (ISSR) have been taken up in 13 silkworm strains. It has been demonstrated that in formativity, sensitivity and speed of the ISSR-PCR can be substantially enhanced by adding fluorescent nucleotides in the PCR mixture and the method has been termed as FISSR-PCR In another study, genetic diversity among 31 silkworm strains was revealed by microsatellites [123]. Molecular evaluation of bivoltine, multivoltine and mutant silkworms have been carried out using RAPD, ISSR and RFLP-STS markers to study the genetic relatedness [113]. These studies have revealed distinct and unique profile that was specific to bivoltine and polyvoltine strains. The results have indicated their potential use not only in understanding genetic relationships but also as powerful tools to generate markers that are linked to the traits of interest in silkworm.

With regard to improvement of yield attributes, marker systems have been extensively used in silkworms. RFLP analysis in Indian silkworm stocks has revealed the close association of six RFLP markers with high shell and two markers with low shell characters. DNA fingerprinting studies to search for markers associated with yield attributes in the silkworm using AFLP markers were taken up by [118]. AFLP molecular linkage maps with a relatively high density for location of QTLs controlling the quantitative traits of silkworm cocoons were constructed using AFLP markers [123, 112, 160].

Among the DNA markers, particularly PCR-based markers like RAPD were being used for genetic analysis of plant and animal genomes. RAPD analysis has several advantages over other DNA markers. These includes relatively shorter time (1-2 days) required to complete analysis after standardization; no need of prior information on the genome of an organism; availability of series of primers for analysis; minimal operational cost requirement; relatively smaller amount (=20ng) of high molecular weight DNA; simpler protocol and involvement of non-invasive sampling for tissue analysis.

RAPD-PCR technique can generate species-specific fragments. These fragments are useful in developing specific 'Sequence Characterized Amplified Region' (SCAR) markers. In contrast to most RFLPs, RAPD markers are usually scored as dominant alleles since a RAPD is present only in one of the parents and amplified in the heterozygote. RAPD markers have enabled a significant advance in the ability to generate linkage maps quickly. Linkage maps of RAPD markers have been reported for Arabidopsis, bananas, lettuce, Eucalyptus, Chicken and so on. RAPD markers were used to screen near isogenic lines to locate bacterial resistance genes in tomato (, down mildew resistance in lettuce, genetic fingerprinting and molecular taxonomy. RAPD was shown as a potential tool in differentiating cryptic mosquito species. RAPD technique was found as a useful tool with great resolving power to discriminate fish species, crab species, oysters. Molecular markers (RAPD) associated with growth, yield and origin of the silkworm was analyzed. This study has helped in identifying a few markers and thereby opened scope of using such marker for incorporating molecular markers in the breeding program for crop improvement in silkworm. Genetic differentiation induced by selection in an inbred population of the silkworm using RAPD and ISSR marker systems has been reported [136]. This investigation has showed that RAPD and ISSR primers can generate polymorphic profiles when amplified with genomic DNA of individuals of longer larval duration (LLD) and shorter larval duration (SLD) lines. The role of RAPD and ISSR markers in silkworm conservation has been elucidated [141]. Further, genetic mapping of Z chromosome and identification of W chromosome- specific markers in the silkworm has been carried out. This mapping has helped in identifying and mapping sex-linked traits in the silkworm. The economic and evolutionary significance of Z- and W-linked genes in silkworm, in particular, and lepidopterans, in general has been noted. Further, RAPD markers linked to densonucleosis refractoriness gene 'nsd-1' has been identified. 'nsd-2' (non-susceptibilty to BmDNV-2) gene was linked to the linkage group 17 of *B. mori* using RFLP markers [149]. 150 RAPD markers were screened for NPV resistance in *B. mori* [126]. Polymorphism and analysis of prothoracotropic hormone and genes of diapauses hormone has been carried out by PCR based primers.

Another class of markers, ISSRs, are DNA fragments of about 100-3000 bp located between adjacent, oppositely-oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10-60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. Because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies. The ISSR markers have been used effectively in studying the genetic relationships among silkworm strains [125]. Srivastava *et al*. [124] analysed the genomic DNA of 15 multivoltine races, using ISSR markers, for studying their the rmotolerance behavior. Chatterjee *et al*. [127] identified ISSR markers associated with productive traits in silkworm.

Application of Isozyme marker system in *Bombyx mori*

Proteins are an important, rather indispensable biomolecules of any organism. The growth and developmental processes of an organism proceed through cascades of expression, repression and interaction of several proteins. Protein-based (isozyme) markers have greatly extended our understanding of metabolic regulation and to understand the differential gene action which is manifested in the form of differential activity of an isozyme during development. Also, isozyme analysis is an important technique for genetic variability studies. Early studies on alpha-and beta esterase isozymes revealed ontogenic variations in *Drosophila*. Similar work was done on *B. mori* using acid and alkaline phosphatase isozymes. Developmental profiles in the isozymes of alpha- and beta-esterases were studied for embryogenesis in silkworm. Genetic polymorphism in 21 bivoltine silkworm races was revealed through isozyme studies. In insects, esterase acts extensively on various kinds of substrate and show high polymorphism and genetic variations. The prospects of using digestive amylase as a marker in silkworm breeding due to its wide genetic diversity, role in better digestibility and higher survival has been highlighted by. To confirm these findings, a breeding scheme was designed and high activity amylase genes from the indigenous polyvoltine di] onor parents were introgressed into the productive bivoltine parents used as recurrent parents and near isogenic lines of the recurrent parents have been developed. Evaluation of hybrids developed by amylase-marker assisted selection has been carried out and on-farm trials have been conducted which indicated the superiority of GEN3xGEN2 hybrid.

Marker system in other organisms

A RAPD screen successfully identified genetic markers for life span in *D. melanogaster* on the basis of large allelic frequency differences between selected and control lines, making it a first step towards identifying QTLs of longevity. The phylogenetic relationship among the members of the *nasuta* group of *Drosophila* has been done using RAPD and ISSR polymorphisms. The investigation has revealed that phylogenetic tree generated by RAPD analysis was nearly in complete congruence with the classification based on morpho-phenotypic characters. Molecular distinction amongst varieties of mulberry was investigated using RAPD and DAMD (Direct Amplification of Minisatellite DNA) profile. The study has shown that RAPD and DAMD data were useful in distinguishing between the nine mulberry varieties. These results were consistent with fact that mulberry was known to have highly heterozygous varieties with larger number of natural hybrids between unisexual mulberry parents.

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