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**EXAMINATION OF TAIL TENDON BREAK TIME: STATUS AS A  
BIOMARKER OF AGING & GENETIC INFLUENCES**

A Dissertation in  
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by

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## ABSTRACT

Tail tendon break time (TTBT) has been considered a biomarker of aging to examine collagen crosslinking changes between strains and species, and also to measure genetic differences with age. Investigation of changes in TTBT with age and genetic influences were examined in two different projects: the Biomarkers of Aging in Mice (BAM) and QTL Analysis of Age-Related Phenotypes (QAARP). The BAM project examined biomarkers of aging (behavioral, physiological and immunological) longitudinally in two generations of a heterogeneous stock (HS) of mice, with measurement occasions at 45, 90, 360, 630, and 900 days of age. Aspects of TTBT that were assessed included correlations across the ages, the relationship with longevity and other biomarkers, and heritability estimates across time determining the genetic influence on TTBT during the lifespan. The QAARP project utilized B6D2F<sub>2</sub> animals from a C57BL/6J and DBA/2J cross, and 23 BxD recombinant inbred (RI) strains derived from the same progenitor strains, evaluating a wide array of phenotypes. In this study, examination of TTBT included calculation of heritability estimates for the B6D2F<sub>2</sub> and BxD RI animals, a QTL analysis in the same two groups of mice, and further examination of two strains: BxD 8 and BxD 22 with unusual TTBT values. The calculated heritability estimates were extremely low for the HS mice throughout the lifespan, whereas the B6D2F<sub>2</sub> and BxD RI animals revealed high estimates of heritability in early life that decreased with age. Furthermore, the correlations of TTBT demonstrated instability across age, which implies TTBT at one time point does not predict subsequent values later in life. The relationships of TTBT with other biomarkers

were inconsistent during the lifespan, differing between ages and sexes, indicating changes in developmental processes across time. Data from both studies show that TTBT does not reliably predict longevity. The QTL analysis nominated eight QTLs, two associated with potential candidate genes, and six that may be informative for other genetic factors affecting the extracellular matrix. The BxD 22 RI strain displays TTBT values much greater than the other BxD RI strains, apparently influenced by a single locus. The other outlying BxD RI strain (BxD 8) exhibits a large significant sex difference of increased TTBT occurring between early adulthood and middle-age, suggesting a potential hormonal difference between this strain and the other BxD RI strains. These analyses of TTBT provide evidence for TTBT as a possible measure of functional aging, and as a relatively independent aging process in Comfort's (1979) hierarchical clock representation of aging.

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## Chapter 1

### Introduction

Tail tendon break time (TTBT), an experimental assay to measure the strength of tail tendon collagen fibers, the primary component of the tendons, has become widely used as an index of non-enzymatic crosslinking of collagen molecules, and has been suggested as a measure of aspects of aging (Verzar, 1964a; Sell & Monnier, 1997).

### Collagen Structure

Collagen, the most predominant protein in connective tissue, is a vital component of body composition from tendons to bones and skin. Collagen is observed in smooth muscles and is found in blood vessels, kidneys, bladder, heart, and the digestive tract.

There are at least 20 types of collagen (Table 1-1) found in fibril and non-fibril forming structures. The fibril-forming collagens include types I, II, III, V and XI, and provide the structural framework for tissues (Kanungo, 1980; Heller, 1987; Silver et al., 2003; Lodish et al., 2000).

Table 1-1: Collagen types and distribution. \*FACIT = fibril-associated collagens with interrupted triple helices (Kanungo, 1980; Heller, 1987; Silver et al., 2003; Lodish et al., 2000)

Collagen Types and Tissue Distribution			
Collagen Type	Constituent $\alpha$ chains	Genes	Major Tissue Distribution
I	$\alpha 1(I)$ , $\alpha 2(I)$	Col1a1, Col1a2	Skin, tendon, bone, placenta, arteries
II	$\alpha 1(II)$	Col2a1	Cartilage, intervertebral disc, vitreous humor
III	$\alpha 1(III)$	Col3a1	Skin, lung, arteries, uterus, liver, stroma
IV	$\alpha 1(IV)$	Col4a1, Col4a2, Col4a5	Basement membranes
V	$\alpha 1(V)$ , $\alpha 2(V)$ , $\alpha 3(V)$	Col5a1, Col5a2, Col5a3	Placenta, skin, chorioamnion
VI	$\alpha 1(VI)$ , $\alpha 2(VI)$ , $\alpha 3(VI)$	Col6a1, Col6a2, Col6a3	Blood vessels, uterus, ligament, lung, skin, kidney
VII	$\alpha 1(VII)$	Col7a1	Chorioamniotic membranes, skin, esophagus
VIII	$\alpha 1(VIII)$	Col8a1, Col8a2	Endothelial tissue
IX	$\alpha 1(IX)$ , $\alpha 2(IX)$ , $\alpha 3(IX)$	Col9a1, Col9a2, Col9a3	Cartilage, vitreous humor, intervertebral disc
X	$\alpha 1(X)$	Col10a1	Cartilage
XI	$\alpha 1(XI)$ , $\alpha 2(XI)$	Col11a1, Col11a2	Cartilage: ear, nose, spine
XII	$\alpha 1(XII)$	Col12a1	Interacts with Type I containing fibrils
XIII	$\alpha 1(XIII)$	Col13a1	Transmembrane collagen
XIV	$\alpha 1(XIV)$	Col14a1	Structural domains
XV	$\alpha 1(XV)$	Col15a1	Eyes, kidneys
XVII	$\alpha 1(XVII)$	Col17a1	Skin: epidermolysis bullosa
XVIII	$\alpha 1(XVIII)$	Col18a1	Endothelial tissue
XIX	$\alpha 1(XIX)$	Col19a1	FACIT* collagen
XXIV	$\alpha 1(XXIV)$	Col24a1	Regulation of type I collagen fibrillogenesis
XXV	$\alpha 1(XXV)$	Col25a1	Beta amyloidogenesis, neuronal degeneration
XXVII	$\alpha 1(XXVII)$	Col27a1	epithelial

Procollagen, the structural unit of collagen, is a long protein consisting primarily of type I collagen. The procollagen unit consists of three coiled subunits composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain. The three chains are twisted around each other into a right-handed triple helix resembling a rope-like structure. Each  $\alpha$  chain is approximately 1000 amino acids long [primarily glycine (Gly), proline (Pro), and hydroxyproline (HYP)] with a repeating Gly-Pro-X, where X can be any amino acid. On the side chain of glycine, an H atom is the only atom that can fit into the center of the three-stranded helix. Hydrogen bonds linking the glycine with a peptide carbonyl group help hold the chains together (Silver et al., 2003; O'Brien, 1992; Lodish et al., 2000).

The non-helical peptide ends of the collagen chain contain the amino acid hydroxylysine. Covalent aldol cross-links are situated between the two lysine or hydroxylysine residues at the C-terminus of one collagen molecule and the two similar residues at the N-terminus of an adjacent molecule. These covalent cross-links stabilize the collagen molecules generating a strong fibril. The fibrils, approximately 50nm in diameter are assembled together in parallel bundles known as collagen fibers. (Lodish et al., 2000; O'Brien, 1992).

Cross-linking of collagen increases with age, resulting in collagen with reduced solubility, and decreased overall flexibility. As a consequence of the increased cross-linking, tendons stiffen, making the tendons less able to sustain applied loads and more susceptible to injury (Selvanetti, et al., 1997).

## **Non-Enzymatic Cross-linking**

The reaction of glycation in collagen is referred to as the Maillard reaction, where advanced glycation endproducts (AGEs) are formed by a reaction of reducing sugars and biological amines (Zieman & Kass, 2004).

This reaction commences when the aldehyde of a reducing sugar, such as glucose, non-enzymatically reacts with a biological amine (amino groups) to produce a Schiff base. This is an equilibrium reaction and is highly reversible and dependent on the concentration of available sugars, with lower concentrations of sugar reversing the reaction, degrading the Schiff base. If glucose is removed or levels are lowered, the unstable Schiff base will reverse back to sugars and proteins within minutes. Schiff bases can go through a rearrangement, forming intermediates, Amadori products, which are more stable than the Schiff base, and like the Schiff base, the amount formed is related to the glucose concentration. Even though more stable than a Schiff base, the Amadori product formed is reversible like the Schiff bases and will attain equilibrium with glucose. Amadori products can in turn, over a period of several months to years, undergo rearrangements and dehydrations forming irreversible AGEs. These endproducts will cross-link proteins inter- and intra-molecularly, thus increasing the amounts of collagen crosslinking occurring within the organism. Enhancement of this reaction can be due to increased glucose concentrations, increased temperature in the organism, and pH raised above 7 in solution (Cerami et al, 1987; Lee and Cerami, 1992; Cerami, 1985, Zieman & Kass, 2004).



AGE accumulations over time contribute to changes in structure and function of tendons, bones, skin, cardiovascular tissue, and smooth muscle tissues in the body. Numerous studies have demonstrated non-enzymatic glycation to be a mechanism of increasing cross-linking with age (e.g. Cerami et al., 1987; Ziemann & Kass, 2004).

## **Tendons**

Tendons, composed of collagen fibers, primarily Type I collagen, are an integral part of the musculotendinous unit. The tendon connects muscle to bone, where it serves to transfer the force of contraction from muscle to bone. In addition, tendons are able to withstand large forces (O'Brien, 1992).

Tendons vary in shapes and sizes dependent upon function in the body. For example, flattened tendons may form a major portion of a muscle such as the external oblique muscle in the anterior abdominal wall. Tendons as observed in tails of mice consist of several collagen fibers and are round in shape (O'Brien, 1992).

## **History**

One of the early pioneers in collagen aging research was Verzar (1957), who applied various physical tests to collagen. He worked with tail tendons as a result of their ease of handling, physical uniformity, and the possibility of extracting many fibers at once. Verzar saw many age-related changes in collagen: appearance of more cross-links

and decreases in solubility with age (Verzar 1964a). The greater number of cross-links resulted in decreased flexibility and increased stiffness of the aged body.

One particular property Verzar (1964b) examined was thermal contraction, the shrinkage that occurs when collagen fibers are heated, resulting in visible effects of reduced density and changes in physical dimensions.

Chemical contraction of collagen fibers has also been used to assess changes in collagen with age. Lyotropic agents (molecules that can be suspended in solution), such as potassium iodide, sodium perchlorate or urea are used instead of heat. Banga (1957) first used potassium iodide and Chvapil and Hruza (1959) utilized sodium perchlorate to induce chemical contraction of collagen fibers.

Verzar (1964b) and Elden and Boucek (1962) studied chemical contraction in a urea solution (a compound that will denature the protein), with the use of urea ultimately serving as a basis for numerous other studies assaying contraction and collagen fiber breakage. The breakage tests, similar to chemical contraction tests, involve attachment of a sufficiently heavy weight to ensure eventual breakage measuring the rupture time of collagen fibers under load in denaturing reagents (e.g. urea).

Boros-Farkas and Everitt (1967) performed both chemical and thermal tests on collagen fibers from the same rats to determine whether the tests measured the same age changes. Some of their tests included chemical contraction and rupture time in urea, with findings of the two tests showing reasonable correspondence to each other.

The urea assay, first suggested by Elden and Boucek (1962) was modified by Olsen and Everitt (1965) for use on rats. Harrison and Archer (1978) modified the assay

for use in mice and demonstrated repeatability of the procedure several times during the lifespan.

Through a series of experimental tests, Harrison and Archer (1978) determined that optimal results were obtained by using a 7 molar (M) urea solution buffered with 0.1M monopotassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and 0.05 M sodium borate ( $\text{Na}_2\text{B}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$ ). The pH of the urea solution was maintained at 7.5 and was sustained at  $45^\circ\text{C} \pm 0.1^\circ\text{C}$  during the assay. Harrison and Archer demonstrated the repeatability of the test using all four tendon bundles in the tail (2 dorsal and 2 ventral tendons).

### ***Genetic Studies***

Further advancements by Harrison et al., (1978) included comparison of different mouse species, *Mus musculus* and *Peromyscus leucopus*, using collagen as an index of aging. *Mus musculus* females displayed fiber break times that increased twice as fast with age as did that of the *Peromyscus* females. This result suggested that cross-linking as assessed by fiber break times reflects biological age of the two species. Harrison and his colleagues noted that at older ages, where no *Mus musculus* survived, the *Peromyscus leucopus* fiber break time increased much more rapidly than at earlier ages, roughly fitting the aging pattern of *Mus musculus*.

Other evidence (Harrison et al. 1978) illustrated collagen cross-linking as a marker of biological age. This conclusion was based on a comparison of the rate of collagen aging in the measured inbred strains, where there was a lack of a correlation between lifespan and rate of collagen aging. In the same laboratory, both the CBA and

C57BL/6 have mean lifespans of 800 to 900 days, though the rate of collagen aging in the CBA mice was greater than the C57BL/6 mice. Harrison et al. (1978) noted that this outcome reflected reproductive lifespan: C56BL/6 ovulate six months longer than the CBA females.

Higgins et al. (1991) examined fiber break time in DBA/2 and C57BL/6 mice, known to differ in longevity (Goodrick, 1975). The shorter lived DBA/2 strain exhibited significantly higher break times than the longer lived C57BL/6 mice at the same chronological ages. The values recorded at 5 months of age in the DBA/2 mice were equivalent to the values recorded at 10 months of age in the C57BL/6 mice.

Heller & McClearn (1992) examined the C57BL/6, DBA/2 strains and the F<sub>1</sub>S, F<sub>2</sub>S and backcrosses generated from these strains. Their results were consistent with previous work with the fiber break times of the DBA/2 strain being greater than the C57BL/6 strain. There was, however, little to no heritability of fiber break time values at 50 or 150 days of age, though it increased to a moderate influence at 450 days of age.

Dietary restriction was implemented on C57BL/6 and DBA/2 mice to examine its effects with age on fiber break time and levels of pentosidine, an advanced glycated endproduct (Sell & Monnier 1997). In comparison to the control *ad libitum* animals, the fiber break times of the dietary restricted animals were lower, and the change with age did not increase at the same rate as the controls. Pentosidine levels were also lower in the dietary restricted animals and did not increase at the same rate as the control animals.

Even though collagen cross-linking has been shown to increase with age as displayed by the increase of tail tendon break time, the relationship of tail tendon break

time with longevity is unclear. Contrary to the findings of Higgins et al. (1991), Harrison et al. (1978) found tail tendon break time did not correlate with longevity.

Analyses in this dissertation look at the status of tail tendon break time as a biomarker of age, the changes in genetic influence across time, and its relationship with longevity. This dissertation will utilize data from two different projects and datasets. The Biomarkers of Aging in Mice (BAM) project, a longitudinal study, permits monitoring the genetic influences during total lifespan of a heterogeneous stock (HS) of mice. The second project, QTL Analysis of Age-Related Phenotypes (QAARP), a cross-sectional study, includes information on young, middle-aged and old-aged mice in both F<sub>2</sub> and recombinant inbred (RI) strains of mice.

### **Biomarkers of Aging Project**

The Biomarkers of Aging in Mice project, a study of aging in genetically heterogeneous mice, assessed two generations longitudinally over a variety of phenotypic domains. Behavioral, physiological, and immunological variables were measured at 45, 90, 360, 630 and 900 days of age. At 45 and 90 days of age, 271 mice were assessed, 251 mice at 360 days of age, 195 mice at 630 days of age, and 75 mice at 900 days of age. For the present analysis, tail tendon break time was examined across age in comparison with the variables included in these three domains. Brief descriptions of the measures follow. More detailed descriptions are provided by Heller et al. (1998).

### **Measures of Behavioral Biomarkers of Aging**

The behavioral domain of the BAM project includes measures of activity, muscle coordination, and autonomic reactivity, measured for every animal at each measurement occasion. Using the File apparatus, adapted from File and Wardill (1975), sector entry activity (factivity), rearing on hind legs (frears) and headpokes (hdpkcs) were recorded for each animal. In a procedure based on the work of Dean et al. (1981), a mouse was placed upon a dowel rod, and the time until the mouse fell off the rod (rod drop) was measured (maximum of 60 seconds). The amount of activity of the mouse on the rod was also measured, recording the number of segments the mouse entered along the rod (rod activity). The behavior tests using the tight wire (cord) were adapted from Miquel and Blasco (1978) and Ingram et al. (1982). The amount of time the mouse grasped the cord with its front paws until it dropped, made its way to the end of the cord, or 60 seconds elapsed, was measured (corddrop). Three trials during each session were conducted for measures on the dowel rod and cord. Observations for each variable were averaged over the three trials to create a single value for each mouse for each occasion.

### **Measures of Physiological Biomarkers of Aging**

Markers in the physiological domain tested an array of biological processes. Tail tendon break time intended to measure collagen aging, was collected for each mouse using one tendon in the tail at each time point starting at 90 days of age. Tests measuring glucose levels (gluc) were performed prior to any testing and again following a 40-minute glucose challenge of an injection of 20 mg% solution glucose (gluc40). In

addition, hematocrit levels at baseline (hema) and again 40 minutes following a glucose injection (hema40) were collected. Urine concentrating ability was tested with assessment of osmolality of a sample (urine) and again following 24 hours of water deprivation (urine24). Lastly, glutathione peroxidase (gshpx) levels in the blood were collected at each measurement occasion for each mouse.

### **Measures of Immunological Biomarkers of Aging**

Levels of concanavalin A (cona), white blood cells (wbc), lymphocytes (lymphs), conjugates (conj), dead target cells (dtar), and natural killer cells (nk25 & nk50) were all collected as part of the immunological domain of the BAM project. The natural killer cells were measured at a concentration of 25:1 (25 lymphocytes to 1 target) and 50:1 (50 lymphocytes to 1 target).

### **QTL Analysis of Age-Related Phenotypes**

The QTL Analysis of Age-Related Phenotypes project was a study of genetic influences across age in an F<sub>2</sub> population generated from C57BL/6J and DBA/2J progenitor strains and in 23 BxD RI strains. The animals in each population were assessed cross-sectionally, with sacrifice occurring at approximately 200, 500 and 800 days for groups of animals within each population.

The mice were measured on variables within domains of behavior, physiology, blood chemistry, neurotransmitter receptors, oxidative stress, and skeletal composition.

The behavior measurements were the same as those collected in the BAM project. The physiology domain consisted of hematocrit, osmolality and tail tendon break time measurements as included in the BAM project. Blood chemistry, neurotransmitter, oxidative stress and skeletal composition domains were also assessed but will not be addressed in this dissertation. In the B6D2F<sub>2</sub> animals, the behavior measures and several of the physiology measures were collected at three occasions at each age, whereas in the BxD RI animals, the measures were collected once. Three measurement occasions for each age group were conducted for measures of behavior, hematocrit and osmolality.

The specific aims for this dissertation are 1) to assess age-to-age correlations of TTBT and to assess at successive ages the heritability of TTBT and its relationship to other biomarkers, 2) to characterize the extremely deviant tail tendon break time in the RI BxD 22, 3) to initiate investigations on the development of the significant sex difference of tail tendon break time in RI BxD 8 animals between 200 and 500 days of age, and 4) to identify QTLs that influence TTBT at 200, 500 and 800 days of age.



## Chapter 2

### Materials and Methods

#### Animals and Conditions

The Biomarkers of Aging project observed two generations of a heterogeneous stock of mice. All mice in the BAM project were born and housed in a barrier facility maintained by the Center for Developmental and Health Genetics (CDHG) at the Pennsylvania State University. Mice of the same litter, and sex were housed four per microisolator cage, upon weaning. All mice were maintained on a schedule of 12/12 hour light/dark cycle at a temperature of  $21^{\circ}\text{C} \pm 2^{\circ}$  with approximately 50% humidity. Mice were fed the NIH 31 diet, and water *ad libitum*.

The animals were followed longitudinally with examinations occurring at approximately 45 and 90 days of age and thereafter tested approximately every 270 days (360, 630 & 900 days of age). With increased age, the number of animals included in analyses decreased as a result of attrition. At 45 and 90 days of age, 271 mice (males & females) were analyzed, at 360 days of age 251 mice, at 630 days 195 mice, and at 900 days of age 75 mice were available. The focus of the dissertation has not included the measurements of animals at 45 days of age, as tail tendon break time (the primary variable of interest) was first recorded at 90 days of age. Mouse tails have four tendon bundles (2 dorsal and 2 ventral), allowing the assay to be repeated only three (1<sup>st</sup> time + 3 repeats) times during the life of the animal.

All mice in the QTL Analysis of Age-Related Phenotypes project (C57BL/6J [B6], DBA/2J [D2], 23 BxD recombinant inbred strains, and B6D2F<sub>2</sub> mice) were born and housed in the same barrier facility under the same conditions as the animals in the BAM project, though these animals were fed autoclaved Purina Laboratory Rodent Chow Diet #5010. The cross-sectional analyses included: 379 mice between 150-200 days, 372 mice between 450-500 days, and 265 mice 750-800 days of age for the B6D2F<sub>2</sub> (F<sub>2</sub> generation of C57BL/6J & DBA/2J cross), the TTBT means for 22 BxD RI strains were derived from 475 mice at 200 days, 385 mice at 500 days, and 230 mice at 800 days of age. The BxD 22 RI strain was removed as an extreme outlier, with TTBT values much greater than four standard deviations from the mean of strain means.

Further exploration from the QAARP project included additional matings of the RI BxD 22 strain with the progenitor C57BL/6J strain. A mating of the BxD 22s to the C57BL/6J parental strain was performed to provide (22)x(B6)F<sub>1</sub> animals. These (22)x(B6)F<sub>1</sub> mice (n = 68) were mated generating F<sub>2</sub>s ((22)x(B6)F<sub>2</sub>) originating from the BxD 22 RI strain and the C57BL/6J mice.

### **Collagen Denaturation Assay (Tail Tendon Break Time)**

The collagen denaturation assay procedure was the same for both projects. In BAM, tail tendon break time measurements were collected at approximately 90, 360, 630 and 900 days of age from the same animals.

At ~200/500/800 days of age in the QAARP project, mice from each age group were sacrificed. Following sacrifice by cervical dislocation, the tails were collected prior to further dissection and harvesting of tissues for additional experimental assays.

The procedure for collagen fiber removal was a modified version of that reported by Harrison and Archer (1978). Tendons were removed from the tail, and individual collagen fibers were separated by forceps in the visual field of a dissecting microscope. Three fibers of intermediate thickness were chosen for each mouse. Each fiber was attached to a 2.0 gram weight using surgical thread, and suspended in a flask filled with a 7M urea solution of a 7.5 pH prepared to denature the collagen fibers. Fiber break times were measured by recording time of initial suspension until the fiber broke, with the weight dropping to the bottom of the flask.

### **Chemical Components Analysis**

The chemical components analysis was completed by Dr. Vuokko Kovanen and colleagues at the department of Health Sciences and Finnish Centre for Interdisciplinary Gerontology at the University of Jyväskylä, Finland.

Collagen fiber samples from DBA/2J and BxD RI 22 animals were freeze dried, and dry weights were taken. The samples were hydrolyzed in 6M hydrochloric acid (HCl) at 108°C for 24 hours. A fixed amount of the hydrolyzate was evaporated to dryness and dissolved into 0.1 M Heptafluorobutyric Acid (HFBA), with 100µl injected into the HPLC (high performance liquid chromatography) apparatus.

Hydroxypyridinoline, deoxypyridinoline, and pentosidine were measured during a single reversed phase HPLC run as described by Bank et al. (1997).

Between 0 to 16 minutes, the wavelength for hydroxypyridinium and pyridinium cross-links was 400nm for emission and 295nm for excitation. From 16 to 60 minutes the wavelength was changed to 328nm for emission and 378nm for excitation for pentosidine. A gradient was built up to contain 17% eluent B (75% acetonitrile with 0.13% HFBA) at 0 minutes and 25% B at 30 minutes for elution of the cross-links. Eluent A was 0.13% HFBA, with a flow rate of 1ml/min. Hydroxypyridinoline eluted at 12 minutes, deoxypyridinoline at 13.5 minutes and pentosidine at 23 minutes.

The concentrations of the cross-links are expressed as picomoles (pmol) per nanomole (nmol) of collagen. The calculations are based on the use of pure compounds of hydroxypyridinoline, deoxypyridinoline, and pentosidine as external standards. The detection limit is 0.4 pmol for the pyridinolines, and 0.05 pmol for pentosidine.

The remaining hydrolyzate was used for analysis of hydroxyproline as described by Creemers et al., 1997. Hydroxyproline, a collagen specific amino acid, is generally used to calculate the concentration of collagen.

The HPLC system used for analysis was a Merck Hitachi, which included a pump (model 655), and A-12 Liquid Chromatograph with a L-5000 LC Controller, and Merck Hitachi's fluorescence detector FL Detector L-7480. Duplicate samples were injected with an AS-4000 Intelligent Auto Sampler.

## **Genotyping**

The B6D2F<sub>2</sub> mice (n=396) in the QAARP study were genotyped for 97 microsatellite markers spaced at ~15 to 20 cM intervals throughout the genome for all mice (Vandenbergh et al, 2003). The DNA for genotyping was extracted from tail-tips collected at weaning. For the 23 BxD RIs, 688 microsatellite markers were selected from the Williams et al. (2001) database.

## **QTL Analyses**

### ***Interval Mapping***

Interval mapping, proposed by Lander and Botstein (1989), is used to estimate the position of a QTL between two genetic markers. Interval mapping was originally based on the maximum likelihood approach, but extensions of the analyses permit use of regression as an alternate method. In the present data, both interval mapping methods were used. The statistical program R/qtl (Broman, 2003) was utilized for interval mapping for the B6D2F<sub>2</sub> data using multiple regression with sex as a covariate allowing for investigation of sex differences. Because R/qtl is inappropriate for RI analysis, QTL Cartographer (Wang et al., 2006) was used for interval mapping in the BxD RIs. This program applied the maximum likelihood approach.

QTLs at particular locations on the chromosome can be displayed on a likelihood map using log of odds ratio (LOD) score plotted against chromosomal position (Falconer & MacKay 1996). A LOD score is an estimate of the likelihood that two loci lie near

each other on the chromosome and with a non-zero probability are transmitted together. A LOD score of 3 or greater generally indicates linkage (Churchill & Doerge, 1994). A 1.5 LOD drop-off interval is a confidence interval for interpretation of QTL positioning, calculated by identifying the centimorgan position on either side of the QTL peak with a LOD score 1.5 less than the peak score.

### **Statistical Analyses**

***Statistical Analyses: Specific Aim#1: To assess age to age correlations of TTBT and to assess at successive ages the heritability of TTBT and its relationship to other biomarkers***

Sibling analyses and parent-offspring regressions were calculated to determine the heritability estimates for tail tendon break time at the four measured occasions during the lifespan of the mice. These estimates were compared to the reported heritabilities in the literature.

Bivariate correlations between tail tendon break time values at each time point were calculated to examine the stability and reliability of this biomarker across age. Furthermore, bivariate correlations between tail tendon break time and the variables in each of the domains of the BAM study were determined to identify any potential relationships that may exist. Lastly, the relationship between tail tendon break time and longevity was evaluated.

***Statistical Analyses: Specific Aim#2: To characterize the extremely deviant tail tendon break time in the RI BxD 22***

One step to further the understanding of the extraordinary tail tendon break time of the RI BxD 22 strain is through examination of the histograms displaying the distribution of the (22)x(B6)F<sub>2</sub> TTBT results. This distribution may indicate whether this trait of extreme break time is based on a single gene and whether its mode of action is dominant or recessive. Furthermore, comparisons of collagen, hydroxypyridinoline, pentosidine and deoxypyridinoline between the BxD 22s and the DBA/2Js was completed using independent t-tests.

***Statistical Analyses: Specific Aim #3: To initiate investigations on the development of the significant sex difference of tail tendon break time in RI BxD 8 animals between 200 and 500 days of age***

The QAARP project demonstrated significant sex differences of TTBT in the BxD 8 RI strain starting between 200 and 500 days of age. The collected data of this strain from the 200 and 500 day measurement occasions were compared, from the behavioral, physiological and structural aspects, searching for additional significant sex differences. Furthermore, the length of the reproductive lifespan of the BxD 8 strain was compared to the other BxD strains in the larger study.

From the accumulated information, a series of further tests was proposed to explore further the phenomenon occurring in the BxD 8 strain. These included collecting data for TTBT at shorter testing intervals between 200 and 500 days of age to identify

when the sex difference originates. Pinpointing when this significant difference begins could enable age-specific procedures to be developed to seek the cause of differentiation.

***Statistical Analyses: Specific Aim #4: To identify QTLs that influence TTBT at 200, 500 and 800 days of age***

Interval mapping in the B6D2F<sub>2</sub>s was conducted on raw data, uncorrected for sex differences, allowing sex to be used as a covariate. Adjustments were made to the BxD RI data to correct for sex differences prior to analyses because sex was not included as a covariate in these analyses. For both the B6D2F<sub>2</sub>s and the BxD RIs, animals with scores exceeding four standard deviations from the respective group mean were removed as outliers. The deviant BxD 22 strain was excluded from the analyses with all individual values within the strain much greater than four standard deviations from the mean of the strain means. Early mortality precluded the inclusion of the BxD 13 strain in the analyses of the 500 and 800 day old mice. Furthermore, the analyses of the 800 day BxD RI mice did not include the BxD 8, 14, 16, and 33 strains because fewer than 4 animals survived to 800 days of age for these strains.

Suggestive and significant thresholds of statistical significance (values corresponding to the 37<sup>th</sup> and 95<sup>th</sup> percentiles) were determined from 10,000 permutations of the data (Churchill & Doerge, 1994). Permutations resulted in the estimates of the thresholds of 3.1 LOD (suggestive) and 4.9 LOD (significant) for the B6D2F<sub>2</sub>s. In the BxD RI data, the threshold estimates for suggestive QTLs were LOD scores of 2.6 for all three ages; the significance threshold at 200 days was 3.9, and at 500 and 800 days of age the threshold was 3.7.



## Chapter 3

### Results

#### Examination of Tail Tendon Break Time in the Biomarkers of Aging Project

Tail tendon break time, one of the biomarkers examined in the BAM project, displays age related changes. Several aspects of TTBT in the BAM study that were examined in these analyses include change with age, correlations across the ages, the relationship with other biomarkers in the study, and the relationship of TTBT with longevity. In addition, the amount of genetic influence on TTBT during the lifespan was assessed through heritability values.

#### Change with Age

As shown in Figure **3-1**, the TTBT values of the parents and offspring increase non-linearly with age, with the parental generation differing from the offspring generation. Two-way ANOVAs show that (See Table **3-1**, Table **3-2**, Table **3-3** & Table **3-4**) the generations were significantly different from one another at all measurement time points [90 (TTBT2), 360 (TTBT3), 630 (TTBT4), and 900 (TTBT5) days of age], whereas the sexes were significantly different at 90 and 360 days of age.

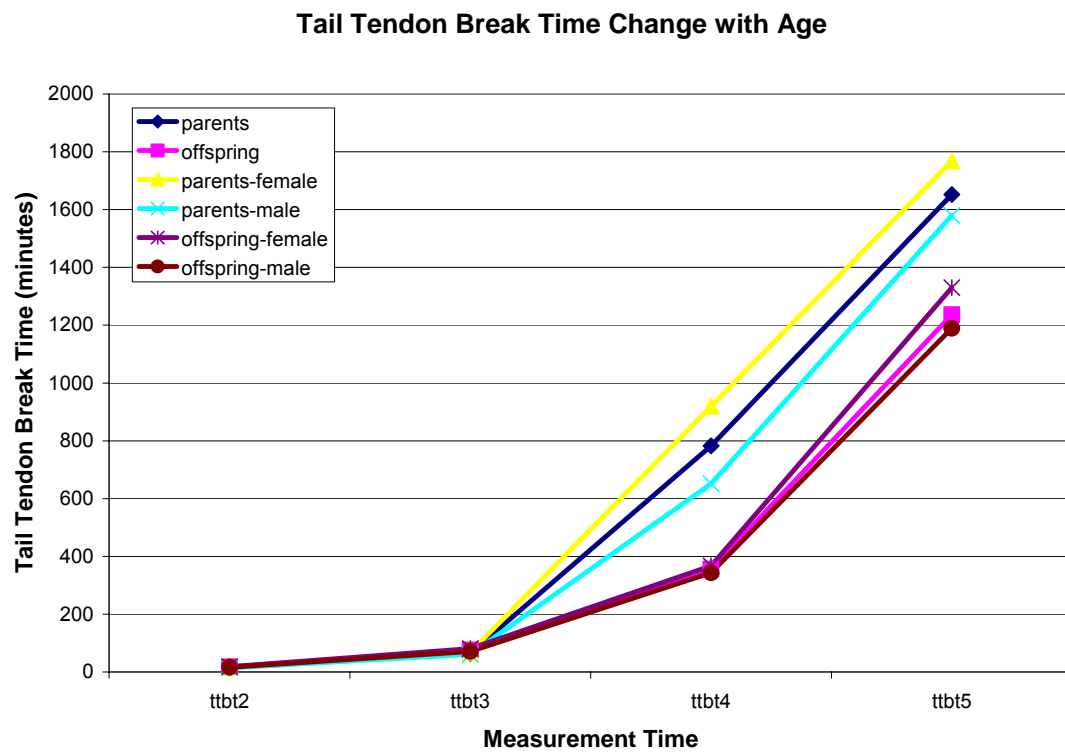


Figure 3-1: Tail tendon break time change across lifespan

Table 3-1: Two-Way ANOVA of TTBT2 using sex and generation as fixed factors

TTBT2

Source	df	F	Sig.
Corrected Model	3	14.012	.000
Intercept	1	4611.157	.000
Xsex	1	19.968	.000
Xgener	1	21.240	.000
xsex * xgener	1	.001	.970
Error	267		
Total	271		
Corrected Total	270		

a. R Squared = .136 (Adjusted R Squared = .126)

Table 3-2: Two-Way ANOVA of TTBT3 using sex and generation as fixed factors

TTBT3

Source	df	F	Sig.
Corrected Model	3	3.483	.017
Intercept	1	1004.287	.000
xsex	1	3.870	.050
xgener	1	6.094	.014
xsex * xgener	1	.097	.755
Error	235		
Total	239		
Corrected Total	238		

a. R Squared = .043 (Adjusted R Squared = .030)

Table 3-3: Two-Way ANOVA of TTBT4 using sex and generation as fixed factors

TTBT4

Source	df	F	Sig.
Corrected Model	3	13.029	.000
Intercept	1	230.512	.000
xsex	1	3.809	.053
xgener	1	32.822	.000
xsex * xgener	1	2.648	.105
Error	181		
Total	185		
Corrected Total	184		

a. R Squared = .178 (Adjusted R Squared = .164)

Table 3-4: Two-Way ANOVA of TTBT5 using sex and generation as fixed factors

TTBT5

Source	df	F	Sig.
Corrected Model	3	2.873	.045
Intercept	1	333.922	.000
xsex	1	1.049	.310
xgener	1	6.670	.013
xsex * xgener	1	.022	.883
Error	51		
Total	55		
Corrected Total	54		

a. R Squared = .145 (Adjusted R Squared = .094)

### Heritability of TTBT in the HS mice

Genetic differences in TTBT between mouse species and strains have been of interest to researchers for over three decades. Harrison and colleagues (1978) observed significant TTBT differences between the *Mus musculus* and *Peromyscus leucopus* species, with a slower rate of change in the longer-lived *Peromyscus leucopus* animals. Additional genetic differences were revealed by Higgins et al. (1991), where the DBA/2 mice showed greater TTBT values than the C57BL/6 mice at the same chronological age. Sell and Monnier (1997) examined dietary restriction and its effects on TTBT, also observing differences between the C57BL/6 and DBA/2 mice. These strain differences provide *prima facie* evidence of genetic influences on TTBT.

Heritabilities for TTBT were calculated for the offspring generation by sibling analysis (Table 3-5). Intraclass correlations were calculated according to Cohen et al. (2003). In Eq. 3.1, the Mean Square between groups ( $MS_{btwn}$ ) and Mean Square within groups ( $MS_{w/in}$ ) were determined through a one-way ANOVA using family as the factor, and the  $n$  value is the total number of animals assessed in the respective measurement group. The value of the numerator represents the variance between the families, whereas the denominator is comprised of the sum of the variance between the families and the variance within the families.

$$\text{Intraclass Correlation} = \frac{MS_{btwn} - MS_{w/in}}{MS_{btwn} + (n-1)MS_{w/in}} \quad \text{Eq. 3.1}$$

This equation can also be written as described by Falconer & MacKay, 1996 in Eq. 3.2, where  $V_A$  is the additive variance component,  $V_D$  is the dominance variance component,  $V_{Ec}$  is the between family environmental variance component, or common environment, and  $V_P$  is the phenotypic variance component.

$$t = \frac{1/2 V_A + 1/4 V_D + V_{Ec}}{V_P} \quad \text{Eq. 3.2}$$

The heritabilities of both the daughters and sons were extremely low from early ages until late life, with a moderate genetic influence in the oldest aged male mice. Although the heritabilities are low, one value was significant in the young females (TTBT2  $p < 0.05$ ), and two were significant in the older males (TTBT3  $p < 0.01$  & TTBT4  $p < 0.05$ ). The higher value of heritability in the males at 900 days of age was not significant due to the small sample size.

The heritabilities of TTBT using parent-offspring regressions were also calculated according to Falconer & MacKay (1996), using mid-parent and mid-offspring values at each age (Table 3-6). There were no significant heritability estimates at any age.

Table 3-5: Calculated heritabilities using intra-class correlations for the daughters and sons in the offspring generation; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; and TTBT5, TTBT at 900 days of age; \*Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.001$

Measurement Time	Daughters	Sons
<i>TTBT2</i>	0.015*	-0.008
<i>TTBT3</i>	0.002	0.047**
<i>TTBT4</i>	0.024	0.077*
<i>TTBT5</i>	-0.141	0.458

Table 3-6: Calculated heritabilities using parent-offspring regressions for the mid-parent and mid-offspring values; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; and TTBT4, TTBT at 630 days;

Measurement Time	Standardized Beta Coefficient	t	Sig.
<i>TTBT2</i>	0.186	0.966	0.343
<i>TTBT3</i>	0.262	1.384	0.178
<i>TTBT4</i>	0.024	0.109	0.914

### Age to Age Correlations

The only consistent significant correlations of TTBT across age are shown in Figures 3-2 & 3-3, which display the correlations of TTBT at 90 days with the other TTBT measurements. The correlation values of TTBT at 90 days with TTBT at 360, 630 and 900 days of age in female mice are 0.236, -0.241, and -0.199, respectively. In the

males, the correlations of TTBT at 90 days with TTBT at 360, 630 and 900 days of age are 0.225, -0.143, and -0.102, respectively. The correlations of TTBT at 90 days of age in the parent generation with TTBT at 360, 630 and 900 days of age are 0.251, -0.025, and 0.154, respectively, and correlations of TTBT at 90 days of age with TTBT at 360, 630 and 900 days of age in the offspring generation are 0.211, -0.075, and -0.179 respectively.

The value of TTBT at 90 days is significantly correlated with TTBT at 360 days in all animals, whether separated by generation or sex. Though significant at  $p < 0.01$  in the females and parents, and at  $p < 0.05$  in the males and offspring, these correlations are small. Furthermore, the correlation of TTBT at 90 days with TTBT at 360 days is positive, whereas the TTBT values at 630 and 900 days are negatively correlated with TTBT at 90 days of age. The correlations of TTBT at 360 with TTBT at 630 and 900 days, and the correlation of TTBT at 630 days with TTBT at 900 days are not significant.



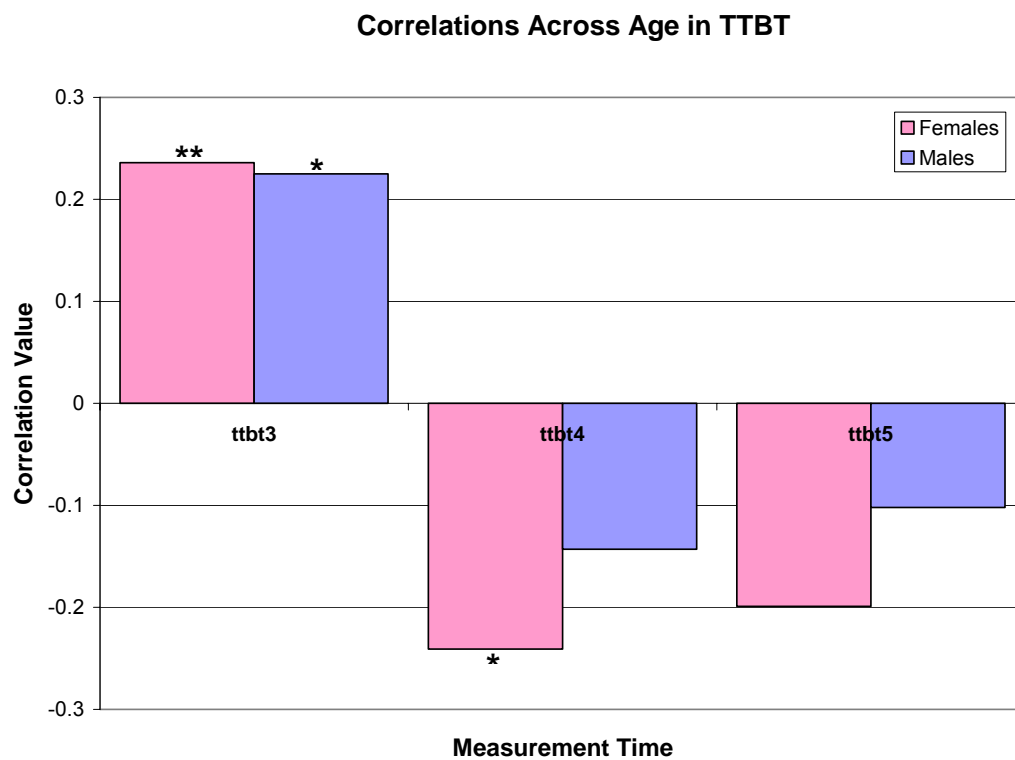


Figure 3-2: Female and male correlations of TTBT at 90 days with TTBT at 360, 630 & 900 days of age; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; and TTBT5, TTBT at 900 days of age; \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.001$

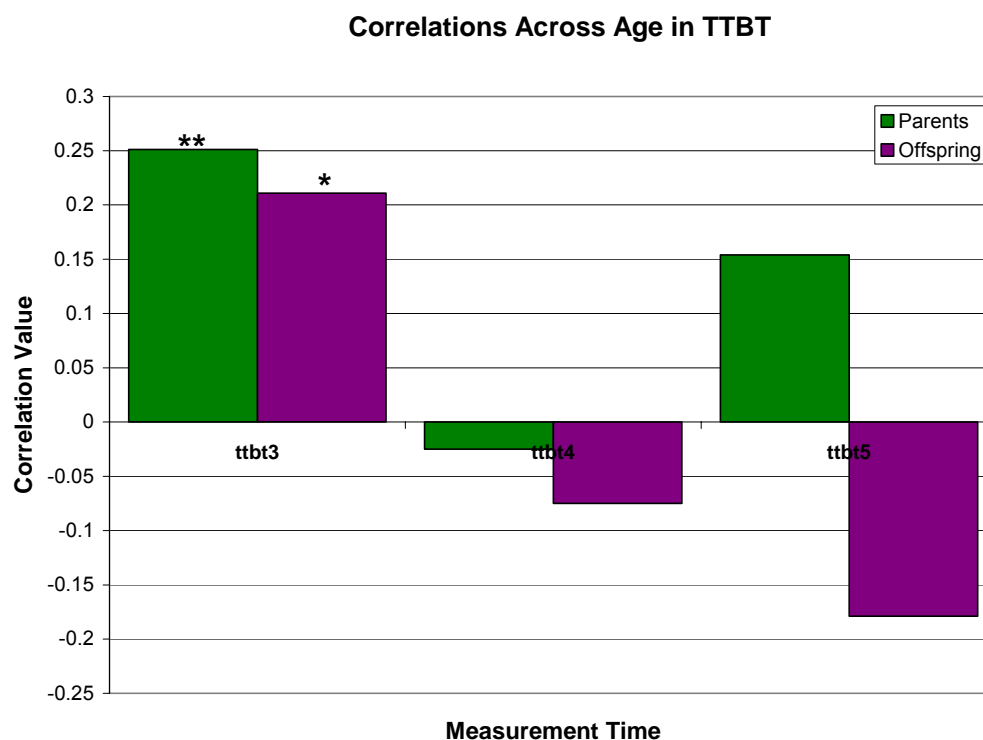


Figure 3-3: Parent and Offspring generation correlations of TTBT at 90 days with TTBT at 360, 630 & 900 days of age; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; and TTBT5, TTBT at 900 days of age; \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.001$

### Relationships to Other Biomarkers

Of the many possible correlations of TTBT with the biomarkers in this study few are found to be significant, and the identified relationships change with age.

Furthermore, most relationships differ between males and females (Figure 3-4, Figure 3-5 & Figure 3-6).

In the females, several of the behavior biomarkers were significantly correlated with TTBT. Sector entry activity in the File apparatus at 630 days of age ( $p < 0.05$ ) was

negatively correlated with TTBT, whereas rod activity at both 360 and 900 days ( $p < 0.01$  &  $p < 0.05$  respectively) was positively correlated with TTBT (Figure 3-4). Cord drop at 360 days ( $p < 0.05$ ) was significant and negatively correlated with TTBT in the males (Figure 3-6). TTBT for both the males and females was positively correlated at 360 days of age for rears on the File apparatus ( $p < 0.01$  &  $p < 0.05$  respectively).

None of the correlations identified for the males and females in the immunological variables were the same in both sexes. TTBT in the females was significantly and positively correlated with lymphocytes at 900 days of age ( $p < 0.05$ ) and negatively correlated with conjugates at 90 days of age ( $p < 0.05$ ) (Figure 3-4). TTBT values were positively correlated with Concanavalin A in the males at 900 days of age ( $p < 0.01$ ), and negatively correlated with natural killer 25 and natural killer 50 at 360 days of age ( $p < 0.05$  for both) in the males. Natural killer 50 was positively correlated at 900 days ( $p < 0.05$ ) in the males (Figure 3-6).

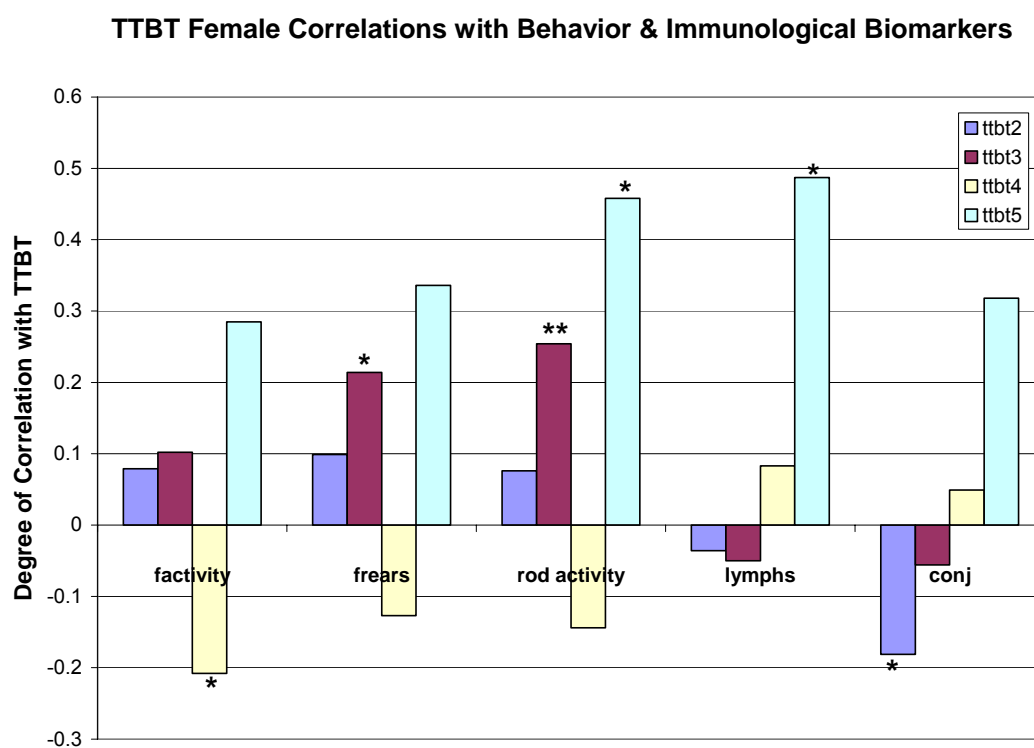


Figure 3-4: Female correlations of TTBT at 90, 360, 630 & 900 days of age with behavior and immunological biomarkers at the same age; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; TTBT5, TTBT at 900 days of age; factivity, sector entry activity; frears, rears; lymphs, lymphocytes; conj, conjugates \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.01$

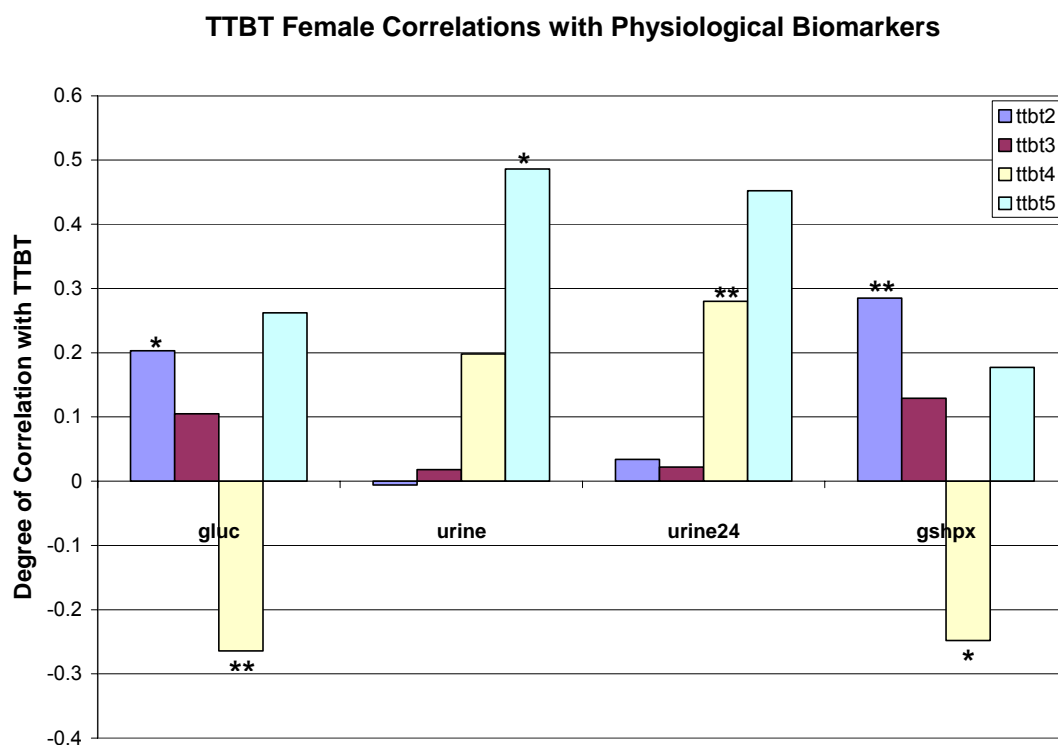


Figure 3-5: Female correlations of TTBT at 90, 360, 630 & 900 days of age with physiological biomarkers at the same age; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; TTBT5, TTBT at 900 days of age; gluc, baseline glucose; urine, baseline urine; urine24, concentrated urine; gshpx, glutathione peroxidase; \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.01$

Significant correlations were found between TTBT and baseline glucose at 90 and 630 days of age in the females ( $p < 0.05$  &  $p < 0.01$  respectively), though the correlation at 90 days is positive, and the one at 630 days of age is negative (Figure 3-5). In the males the correlation of TTBT at 90 days with baseline glucose is positive and significant ( $p < 0.05$ ) (Figure 3-6). Furthermore, both males and females show significant relationships with glutathione peroxidase at 90 and 630 days in the females ( $p < 0.01$  and

$p < 0.05$ ) and at 90, 630 and 900 days of age in the males ( $p < 0.01$ ,  $p < 0.05$  &  $p < 0.05$  respectively). The correlations in both sexes are positive at 90 and 900 days of age and negative at 630 days of age. Correlations of TTBT with baseline urine at 900 days of age ( $p < 0.05$ ), and concentrated urine (urine24) at 630 days of age ( $p < 0.01$ ), were significant and positive in the females.

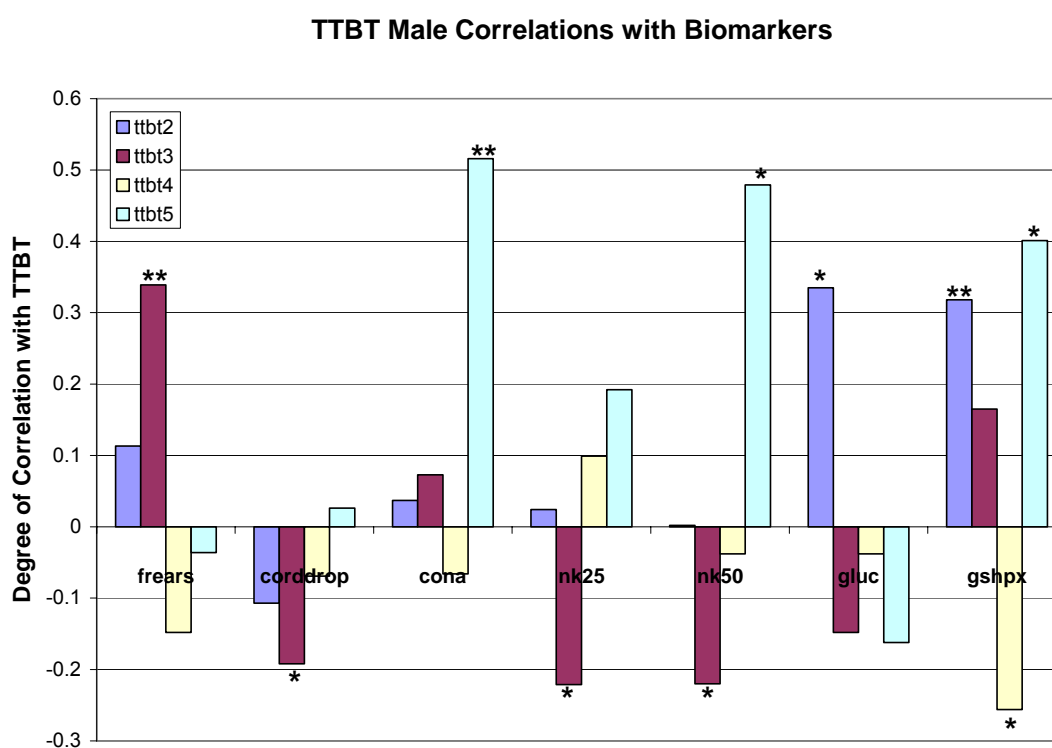


Figure 3-6: Male correlations of TTBT at 90, 360, 630 & 900 days of age with behavior biomarkers at the same age; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; TTBT5, TTBT at 900 days of age; fears, rears; cona, concanavalin A; nk25, natural killer25; nk50, natural killer50; gluc, baseline glucose; gshpx, glutathione peroxidase; \* Significant at  $p < 0.05$ ; \*\* Significant at  $p < 0.01$

### **Correlation with Longevity**

Of the various criteria for biomarkers of aging (Arking, 1991), TTBT clearly complies with that of measurable change with age. The relationship with longevity, another criterion of a biomarker of aging, is regarded by many as of crucial importance. In the current data (Figure 3-7), longevity correlates only with TTBT of females at 900 days of age, but not of males.

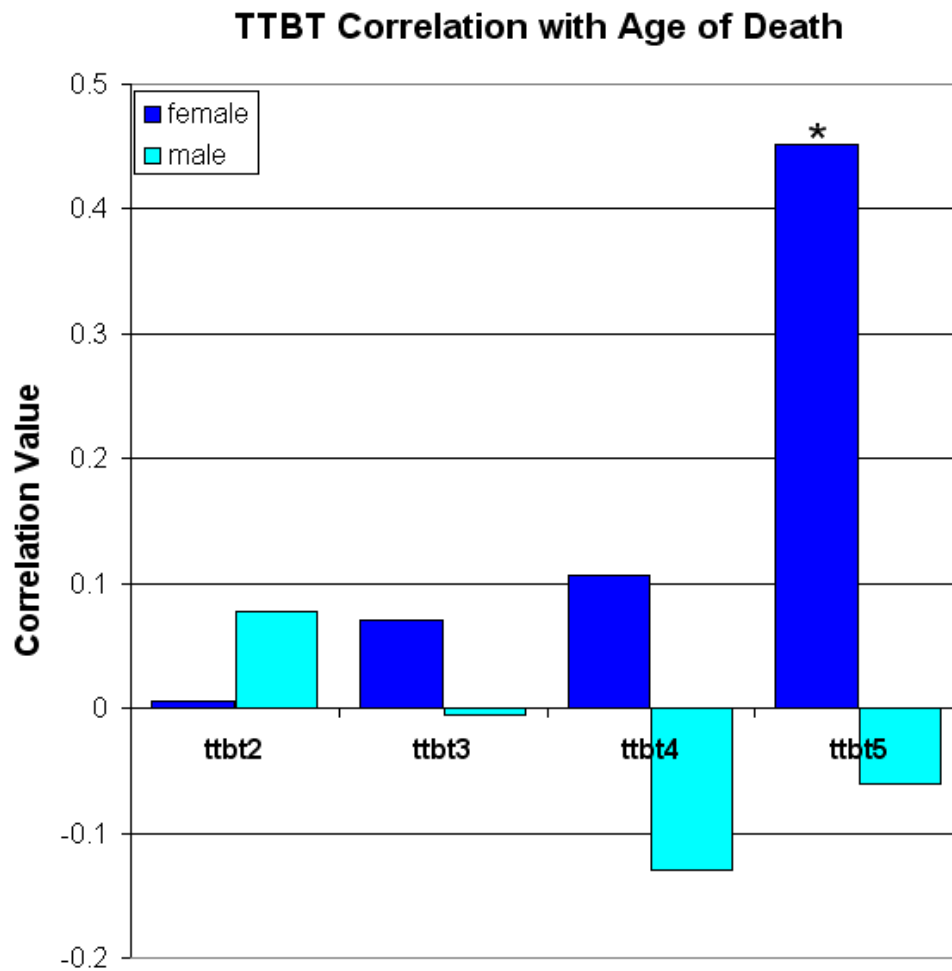


Figure 3-7: Female and male correlations of TTBT at 90, 360, 630 & 900 days of age with age of death; TTBT2, TTBT at 90 days; TTBT3, TTBT at 360 days; TTBT4, TTBT at 630 days; and TTBT5, TTBT at 900 days of age; \* Significant at  $p < 0.05$



## Discussion

### Change with Age

The change of TTBT with age was consistent with results in the literature, but the significant difference between the generations as TTBT increased with age is unexplained. At all ages, TTBT of parents was significantly higher than that of their offspring. This result has not been reported in the literature, and does not have an obvious explanation. The environment for both generations was the same highly controlled barrier facility with all aspects of husbandry the same, eliminating these environmental factors as components causing the significant generational difference of the TTBT values. These generational differences may be the result of susceptibility to subtle environmental influences.

### Heritability Across Time

The low heritabilities from both analyses indicate a small, or absent genetic influence on TTBT. The calculated heritabilities are similar to those found by Heller & McClearn (1992). They studied inbred strains (C57BL/6 and DBA/2), and derived generations, and in young animals reported heritabilities slightly greater than zero. However, they estimated a moderate genetic influence in middle aged mice (~400 days of age), whereas the heritabilities in this study, estimated by sibling analysis, did not increase in the females but increased in late life in the males. The calculated heritabilities from the parent-offspring regressions did not increase with age, and differed from the

results of the Heller & McClearn study. Although the heritability values (narrow-sense) are near zero, these results do not indicate minimal genetic influence on TTBT. These heritability values do not include potential epistatic effects that may affect the genetic influence on TTBT. There is no obvious explanation for this difference between the studies, as rearing and testing conditions for both studies were the same. The restricted gene pool represented in the Heller & McClearn study ( $F_1$ s &  $F_2$ s of the same progenitor strains), and the much greater allelic diversity of the HS mice may account for this difference in heritability values.

### **Correlations Across Ages**

As seen in Figures 3-2 & 3-3 the age to age correlations for TTBT reveal little stability across ages, suggesting individual difference in the factors influencing TTBT change across age. These results suggest that there are substantial developmental changes across age that may be due to genetic or environmental influences on TTBT, and presumably these changes are in the extracellular matrix as aging progresses.

### **Relationships with Other Biomarkers**

Examining the relationships of TTBT to other presumptive biomarkers of aging provides an approach to characterize age-related processes, such as the influence of TTBT on behavioral, physiological or immunological aging as measured in this study. The few significant correlations of TTBT with the other measured biomarkers that were

revealed here are not consistent, differing between sexes and across ages. This outcome suggests heterogeneity of aging and functions during the lifespan. In particular, these results indicate a multi-factorial nature of aging processes, consistent with Comfort's hierarchical clock theory of aging (1979), where TTBT represents a relatively independent clock in the hierarchical system.

### **Correlation with Longevity**

Although TTBT has been designated a biomarker of aging, it has not been found to predict longevity regarded by some as a requirement of a biomarker of aging (Baker & Sprott, 1988; Arking, 1991). TTBT does meet many of the other characteristics of a biomarker; e.g. change with age and repeatability during the lifespan. Harrison et al. (1978) found TTBT not to be a predictor of longevity, which is consistent with this study. These results can be interpreted to mean that TTBT may be a measure of functional aging (affecting functional ability of an individual with age) rather than time until death, further supporting the notion that TTBT represents a relatively independent biological clock in the hierarchical causal system of aging processes.

## Chapter 4

### Examination of Tail Tendon Break Time in the QTL Analysis of Age-Related Phenotypes Project

The QAARP project utilized B6D2F<sub>2</sub> animals resulting from matings of reciprocal F<sub>1</sub> animals derived from the C57BL/6J and DBA/2J strains. Also studied were twenty-three BxD RI strains derived from the C57BL/6J and DBA/2J progenitor strains through repeated brother-sister matings. The F<sub>2</sub> animals are a heterogeneous group of animals, while each of the RI strains are inbred and assumed to be homozygous at every locus. These groups thus represent heterogeneous and homogeneous samples from the same gene pool.

#### Descriptives of the B6D2F<sub>2</sub> and BxD RI samples

Among the B6D2F<sub>2</sub> intercross mice, mean TTBT increased non-linearly with age from 200 to 800 days of age (Figure 4-1). The rates of TTBT change for the progenitor strain means differ (C57BL/6J & DBA/2J), with the mean F<sub>2</sub> rate of change falling between the two parental strains. The female means increase at a slower rate than those of the males (Table 4-1).

### Tail Tendon Break Time

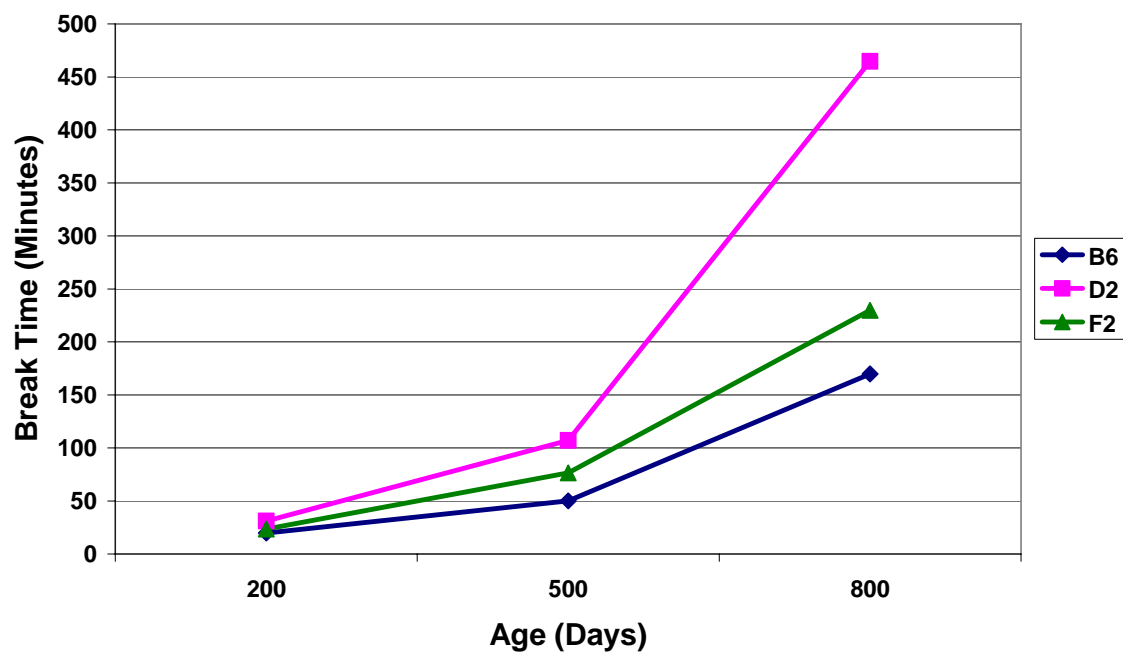


Figure 4-1: Mean TTBT change with age of the C57BL/6J, DBA/2J and B6D2F<sub>2</sub> animals at 200, 500 and 800 days of age. B6: C57BL/6J; D2: DBA/2J; F<sub>2</sub>: B6D2F<sub>2</sub>. The mean values for 200, 500 & 800 days respectively are: B6s: 19.6, 50.14, 169.83; D2s: 30.99, 107.08, 464.55; F<sub>2</sub>: 23.46, 76.51, 229.80

Table 4-1: Tail tendon break times for males and females in the B6D2F<sub>2</sub> intercross at 200, 500 and 800 days of age

	Females			Males		
	200d	500d	800d	200d	500d	800d
B6D2F <sub>2</sub>	23.4 ± 4.7	76.4 ± 22.5	229.8 ± 98.2	24.3 ± 4.4	83.0 ± 26.5	285.9 ± 152.1
<i>n</i>	187	184	120	192	188	145

Distributions of the RI strain means (except for the extreme outlier BxD 22 animals) at different ages are presented in Figure 4-2, Figure 4-3 and Figure 4-4. The continuous variability without distinctly differing categories suggests polygenic rather than major locus influence.

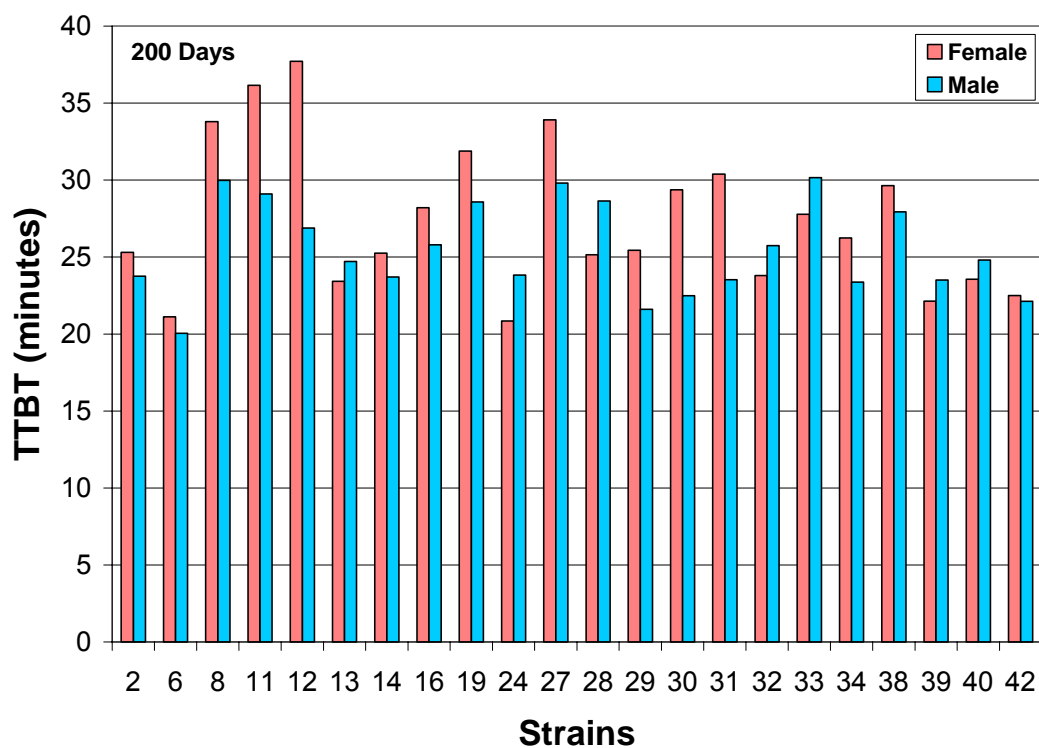


Figure 4-2: Distributions of strain means of tail tendon break time (TTBT) at 200 days of age in the BxD Recombinant Inbred strains (BxD RIs). ■ = Females ■ = Males

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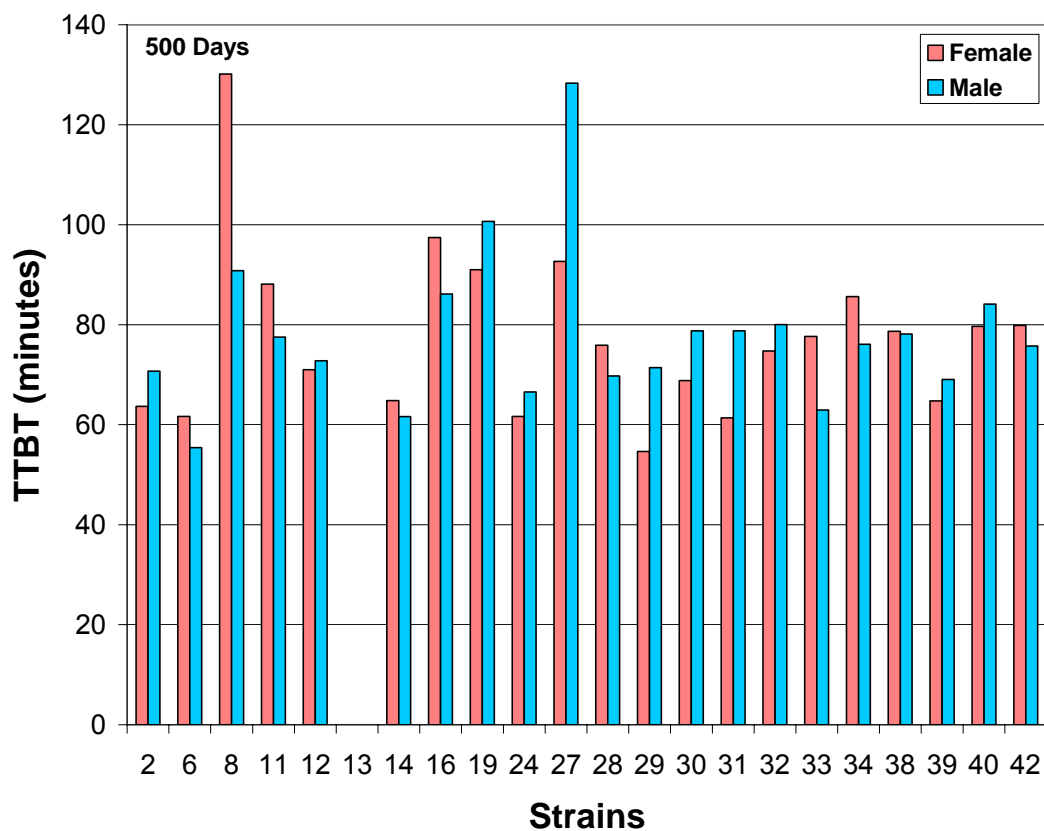


Figure 4-3: Distributions of strain means of tail tendon break time (TTBT) at 500 days of age in the BxD Recombinant Inbred strains (BxD RIs). Strains with no values did not have survivorship at age of testing. ■ = Females ■ = Males

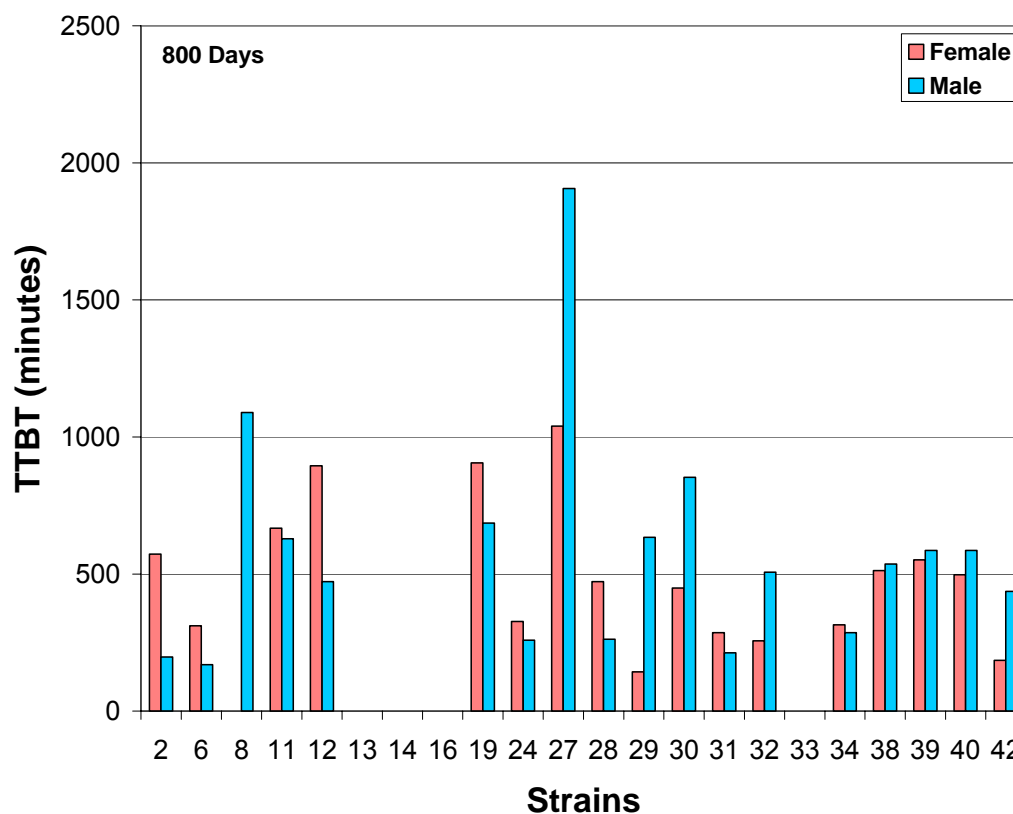


Figure 4-4: Distributions of strain means of tail tendon break time (TTBT) at 800 days of age in the BxD Recombinant Inbred strains (BxD RIs). Strains with no values did not have survivorship at age of testing. ■ = Females ■ = Males

### Heritabilities

Broad sense heritabilities of TTBT were calculated for the RI animals with the outlying BxD 22 strain removed. Heritability in the BxD RIs ( $h^2_{RI}$ ) was estimated by ANOVA by strain as the  $SS_{\text{between strains}}/SS_{\text{total}}$  ( $SS = \text{Sum of Squares}$ ) (Falconer & MacKay, 1996).



The estimates of heritability for TTBT calculated for the BxD RI mice for males and females, respectively, are 0.35 and 0.55 at 200 days of age, 0.38 and 0.69 at 500 days of age, and 0.47 and 0.35 at 800 days of age.

### **Correlations**

In the B6D2F<sub>2</sub> animals, the TTBT of young adults (150-200 days of age) correlated with both sacrifice and adipose fat weights, whereas cord drop means 2 and 3, extensor digitorum longus weight and hematocrit 3 were significantly correlated with TTBT in the middle-aged (450-500 day old) mice. In the B6D2F<sub>2</sub>s, TTBT was significantly correlated with the weights of the soleus and gastrocnemius muscles (See Table 4-2).

The analyses of the correlations in the BxD RIs utilized the means of all RI strains, (except for the BxD 22s). Fewer significant correlations were observed in the BxD RIs than in the B6D2F<sub>2</sub>s between TTBT and the measured phenotypes: three in the youngest age group (150-200 days of age): headpokes, squares and rod sector mean, one in the middle aged group (450-500 days of age): soleus weight, and none in the oldest group (750-800 days of age) (Table 4-3).

Table 4-2: Correlations of TTBT with measured Phenotypes in B6D2F<sub>2</sub> animals; Wt, Weight; Ex. Digit. Long Wt, Extensor Digitorum Longus Weight; \* Significant at p<0.05 level

Age	Phenotype	Correlation
150	Sacrifice weight	0.105*
	Adipose fat weight	0.113*
450	Cord drop mean2	-0.126*
	Cord drop mean3	-0.132*
	Hematocrit3	-0.132*
	Ex. Digit. Long Wt	0.120*
750	Soleus Weight	-0.147*
	Gastrocnemius Wt	-0.144*

Table 4-3: Correlations of TTBT with measured phenotypes in strain means of BxD RI animals; \* Significant at p<0.05 level

Age	Phenotype	Correlation
150	Headpoke	-0.482*
	Squares	-0.448*
	Rod Sector Mean	-0.483*
450	Soleus Weight	-0.518*
750	No correlations	

## Discussion

The heritability values of TTBT decreased with age, indicating an increased amount of environmental influence on TTBT with time. These results differ from those reported by Heller & McClearn (1992), where the narrow-sense heritabilities were minimal and almost non-existent in early life, but increased to moderate correlations nearing middle age. The moderate correlations reported by Heller & McClearn were lower than the broad-sense heritabilities calculated at middle age for this project. In addition, the calculated heritability estimates in this study were much higher than those determined in the BAM study, which were near zero or absent during the entire lifespan of the mouse.

There is no obvious explanation of these differences in heritability estimates, as rearing and testing conditions were the same in all three studies. The differences between genetically segregating populations and inbred populations offer scope for differing influence of Gene x Gene interactions and Gene x Environment interactions. Nevertheless, the continuous distribution of the B6D2F<sub>2</sub>s and the strain differences among the RIs clearly indicate genetic influence on TTBT. The present study offers an intermediate approach to the genetic mechanisms by a search for QTLs.

## Chapter 5

### Examination of Outlier Strains from the Tail Tendon Break Time Analysis

In the QAARP study, two extreme outlier strains were identified. These are discussed separately in further detail, and hypotheses for subsequent studies will be provided.

### Exploration of Sex Differences in the BxD 8 RI Strain

A significant sex difference developed between 200 and 500 days of age in the BxD 8 RI strain (Table 5-1). The females of the BxD 8 RI strain did not live to 800 days, (the age of the last test group), though several male mice did. Therefore, the extent of the sex difference cannot be calculated at this latter age.

The extraordinary differentiation between sexes in this strain and the difference of the females from the other BxD RI strains indicates a potential link between TTBT and longevity.

No strong significant sex difference was evident in 22 of the other strains (Figure 5-1). (The BxD 22 strain was eliminated as an extreme outlier to be described later, and TTBT values could be collected only at 200 days in the BxD 13 strain because of their early mortality.)

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Table 5-1: Mean TTBT values for Males and Females at 200 and 500 days of age for the BxD 8 RI strain; TTBT in minutes

<b>Age</b>	<b>TTBT <math>\pm</math> S.E.</b>
<b>200 days</b>	
Females ( $n=11$ )	33.80 $\pm$ 1.77
Males ( $n=12$ )	29.99 $\pm$ 1.47
<b>500 days</b>	
Females ( $n=8$ )	298.67 $\pm$ 50.71
Males ( $n=9$ )	90.09 $\pm$ 12.02

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### Mean Tail Tendon Break Times

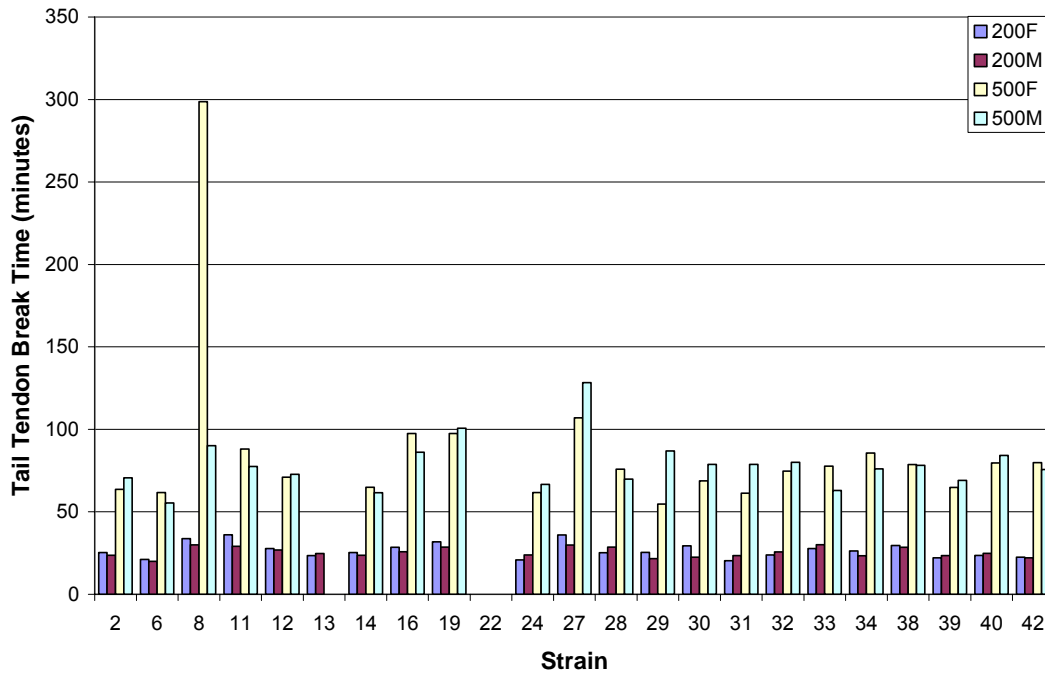


Figure 5-1: BxD RI strain distribution comparing TTBT values at 200 & 500 days of age. 200F, Females at 200 days of age; 200M, Males at 200 days of age; 500F, Females at 500 days of age; 500M, Males at 500 days of age; B6, C57BL/6J; D2 DBA/2J; BxD 22 removed; BxD 13 early mortality

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Insufficient data are available to determine the cause of this significant sex difference, but there are some suggestive correlates. Significant sex differences were observed in the BxD 8 RI strain in phenotypes associated with weight (i.e. body weight at time of sacrifice and weights of the soleus, extensor digitorum longus, and gastrocnemius muscles) (data not shown). Male mice are commonly larger in size than females. The significant sex differences in these weight-related phenotypes are present in animals of all strains, and are not specific to the BxD 8 RI strain. Therefore muscle and body weights

are not exceptional, and can be eliminated as significant differences of the BxD 8 RI strain from the other BxD RI strains.

In the QAARP study, a separate group of the RI strains was raised from birth until death. The survival curves of the males (n=12) and females (n=10) in the BxD 8 RI strain are unremarkable in context of the other strains. Figure 5-2 reveals a difference between the sexes, though the mean longevities do not differ significantly. At approximately 450 days of age, there is a cross-over where the percentage of females surviving starts to decrease faster than the males: this trend continues for the remainder of the lives of the animals of both sexes.

This feature is of particular interest because the significant increase of TTBT in the females is seen at approximately the same age of the survival cross-over.

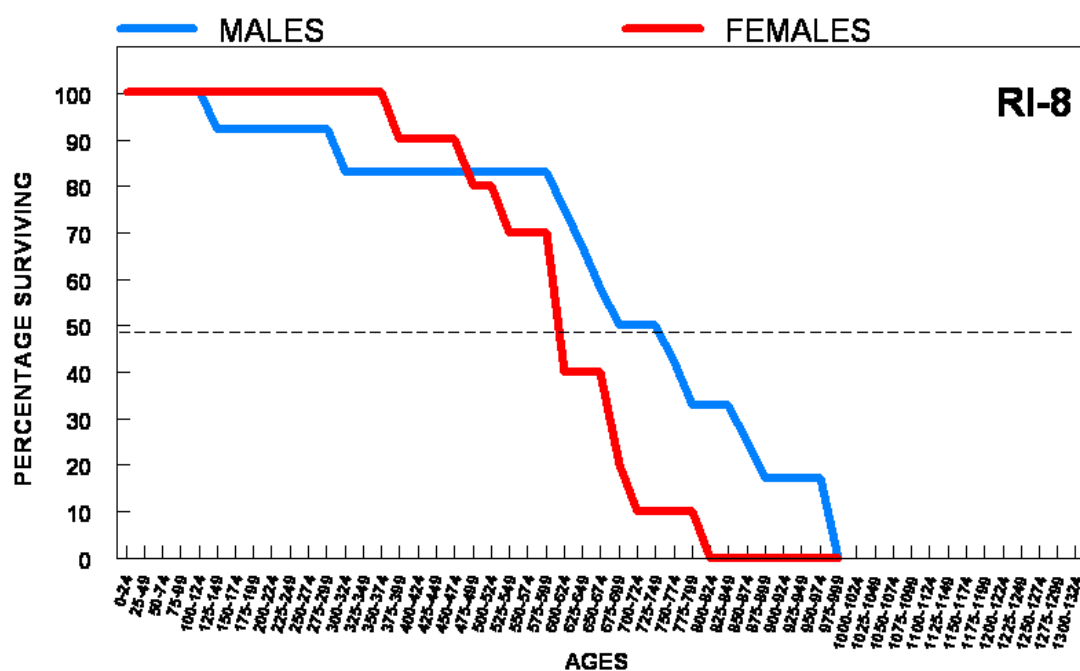


Figure 5-2: Survival Curves of BxD 8 RI strain

### Future Directions for BxD 8 RI strain

Several experimental procedures will be suggested which might identify the causes of the sex difference, and assess whether these factors have a significant impact on other aspects or dimensions of aging of the mouse.

Pinpointing the age of onset of this sex difference is an obvious early step. Examining the tail tendon break time at shorter time intervals between 200 and 500 days of age, such as 100 day increments (200, 300, 400, & 500 days of age) would allow a more precise estimation of the time when the sex differentiation begins.



The unique sex differentiation in the BxD 8 RI strain suggests the possibility of hormonal influence on TTBT. One useful approach might be to test hormone levels of estrogen, progesterone and other sex hormones in the BxD 8 RI strain and compare them to the other BxD RI strains to determine whether there are significant differences present. If significant differences exist, the age of onset of fertility, the average number of litters each female has, the size of the litters, and the age span over which these animals reproduce, in comparison to the other BxD RI strains, would suggest such a hormonal involvement.

An additional approach would be to perform ovariectomies at an early age to reduce the amount of estrogen and other androgens produced in the BxD RI females. Tail tendon break time values could be collected in these ovariectomized animals at middle age (approximately 500 days), and compared to those TTBT values of a control group. A reduction in the rate at which TTBT increases in operated animals would further implicate hormone changes in this exceptional developmental effect.

One more factor that should be examined is body temperature of the BxD RI animals. For example, Harrison and Archer (1978) reported collagen fibers from warmer parts of the tail aged more quickly than those fibers from cooler parts of the tail, and the difference between warmer and cooler fibers increased in older animals. One explanation for variation in TTBT may therefore relate to differences in body temperature. The body temperature and respective tail temperature of the female BxD 8 RI animals may be greater than in other BxD RI strains, therefore increasing TTBT values. A detailed study of the body temperatures and corresponding TTBT values of inbred strains would be informative.

These proposed observations and procedures are a starting point for examining the significant sex difference of TTBT in the BxD 8 RI strain. From the results of these observations and assays, new questions will likely arise to direct future research to understand the nature and significance of this difference.

### **An Exceptional Outlier of Tail Tendon Break Time: BxD 22**

The BxD 22 RI strain exhibited an extreme tail tendon break time. An 11 fold difference of tail tendon break time was present at 200 days of age in comparison to the mean of the remaining 22 strains measured (mean<sub>22</sub> = 290.5 minutes vs mean<sub>RI<sub>s</sub></sub> = 26.1 minutes). At 500 days of age, mean of the BxD 22 RI strain was 19 times higher than the mean of the remaining RI strains (mean<sub>22</sub> = 1645.3 minutes vs mean<sub>RI<sub>s</sub></sub> = 82.0 minutes) (Figure 5-3). Further testing of the BxD RI strains examined at 60 days of age revealed that tail tendon break time in the BxD RI 22s (mean 63.8 minutes) was already 16 times greater than that of the remaining 22 RI strains (mean 4.0 minutes) (Figure 5-4).

The extreme break time at all recorded ages for the BxD 22 RI strain prompted an initial genetic investigation. BxD RI 22s were mated to the C57BL/6J parental strain, and the resulting F<sub>1</sub>s were mated to generate F<sub>2</sub>s. Tail tendon break times were obtained for these F<sub>2</sub> mice (ages 60 to 77 days); the results are consistent with a Mendelian interpretation of a single gene influence on the trait (Figure 5-5). This distribution is consistent with a dominance mode of gene action, with 10 out of 47 of the animals

having categorically lower tail tendon break times than the remaining 37 animals. These 10 animals are presumably homozygous recessive for the non-deviant allele.

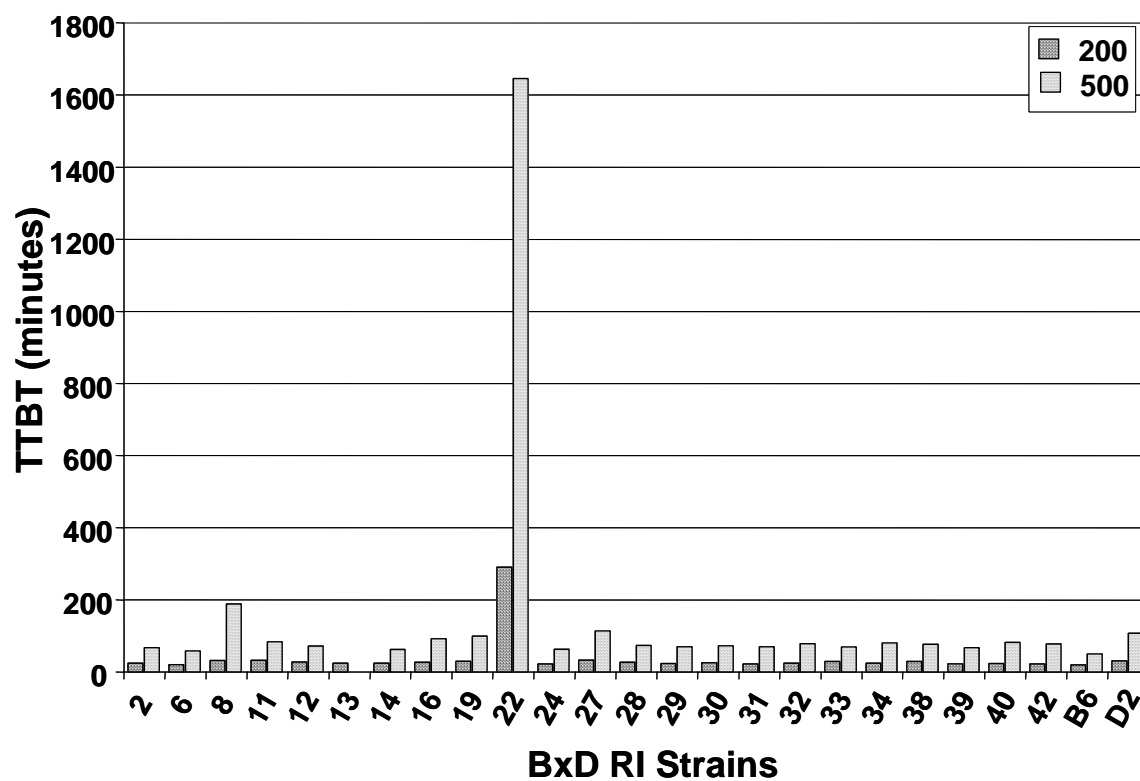


Figure 5-3: TTBT means for DBA/2J (D2), C57BL/6J (B6), and 23 RI BxD strains at 200 and 500 days of age.

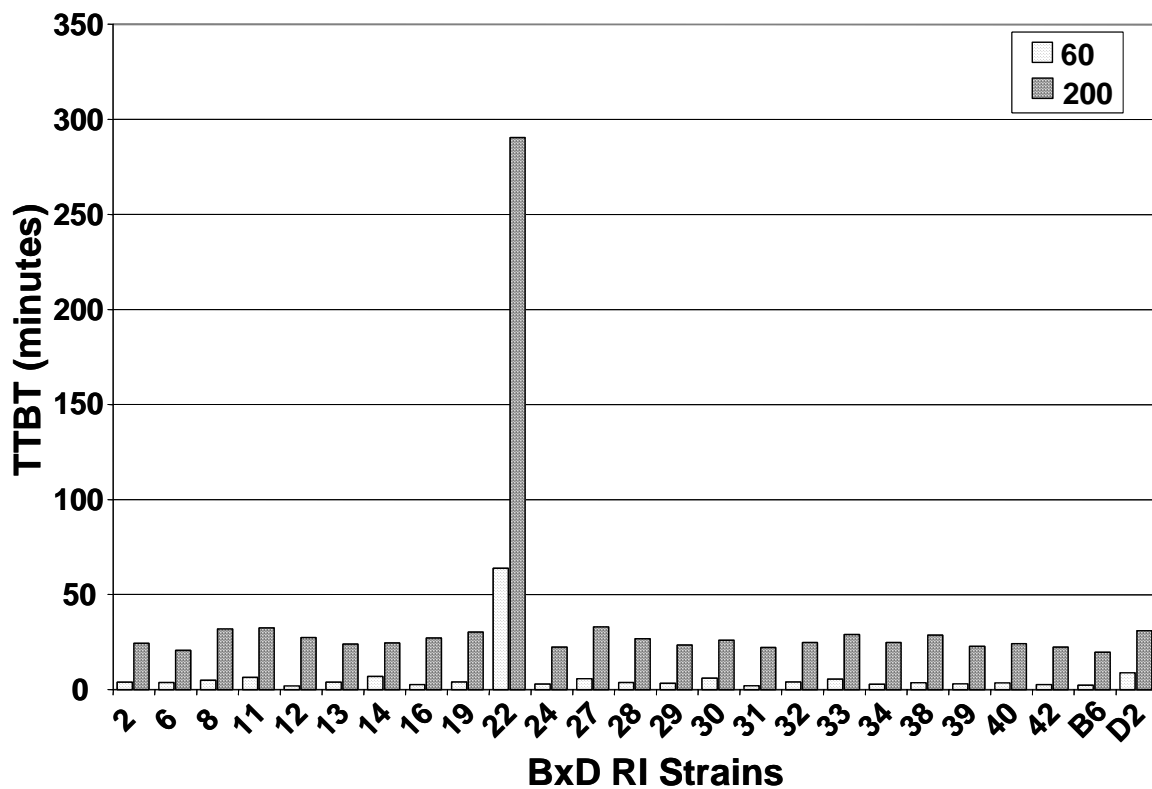


Figure 5-4: TTBT means for DBA/2J (D2), C57BL/6J (B6), and 23 RI BxD strains at 60 and 200 days of age.

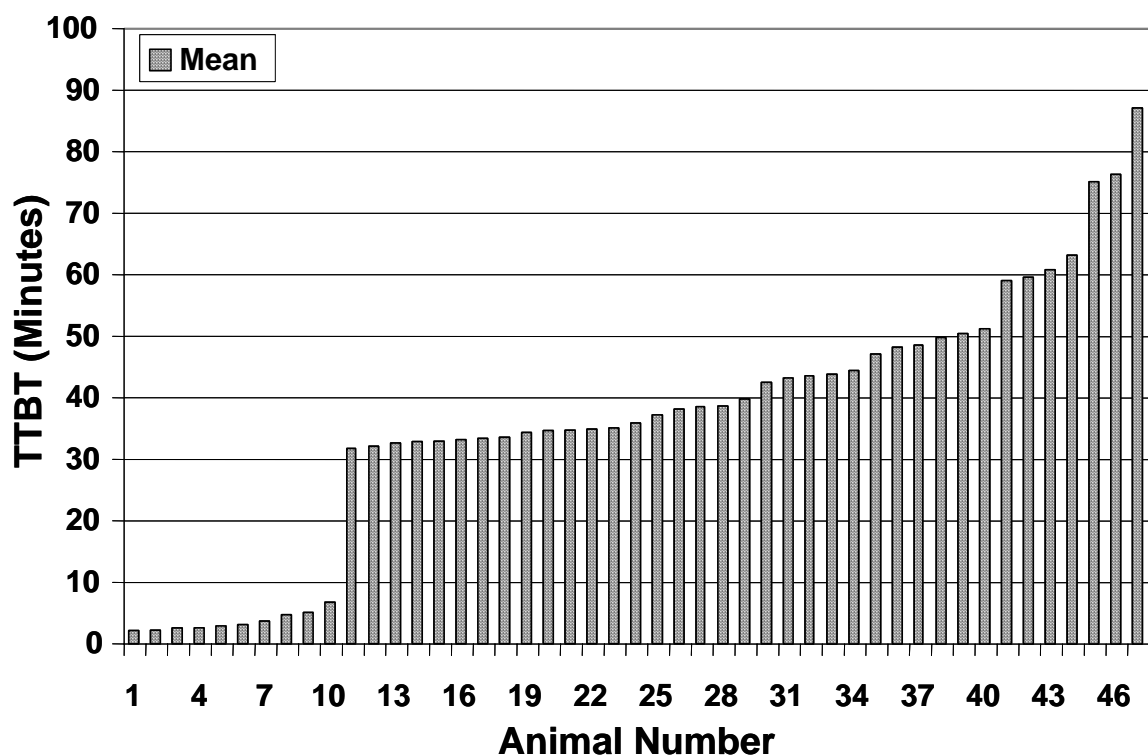


Figure 5-5: TTB T distribution of F<sub>2</sub> mice resulting from RI BxD 22 and C57BL/6J matings.

### Chemical Component Analysis

Preliminary chemical analyses investigated concentrations of collagen, hydroxyproline, and pentosidine, the latter being two different collagen cross-linking compounds, in tail tendons of the progenitor DBA/2J and BxD 22 RI strains. Tendon collagen concentration appeared to be higher in both the male and female BxD 22 RI strain in comparison to the DBA/2J parental strain regardless of age. Both hydroxyproline and pentosidine concentrations were lower in the BxD 22 mice than the DBA/2J mice for both males and females (Table 5-2).

Table 5-2: Collagen, hydroxyproline and pentosidine concentration for male and female RI BxD 22 and DBA/2J mice at young and middle ages.

Group	Sex	Age	Collagen Conc.* Tot Col. mg/dry wt mg	HP/Collagen pmol/nmol	Pentosidine/Collagen pmol/nmol
BxD 22	♂	63	0.043	27.204	0.1671
		63	0.049	10.874	0.1025
		225	0.031	109.768	0.1627
		337	0.049	106.485	0.096
	♀	461	0.028	218.703	0.4182
		63	0.027	19.440	0.1401
		217	0.042	48.342	0.2374
		217	0.036	72.320	0.2772
DBA/2J	♂	61	0.025	12.714	0.1897
		213	0.014	204.162	0.5538
		213	0.013	232.789	0.648
	♀	61	0.020	15.294	0.2665
		213	0.018	137.123	0.6437

\* Collagen Concentration: Total collagen milligrams/dry weight milligrams; HP, hydroxyproline; pmol, pico-moles; nmol, nano-moles

### Discussion of BxD 22 RI strain

These results do not contradict the polygenic interpretation of the “normal” range of variation, but they clearly show that at least one locus has an allelic variant (possibly “rare”), that has an enormous influence on the phenotype. The 3:1 phenotypic ratio describes one potential gene that is influential on TTBT in this deviant BxD RI strain.

The second major constituent of the analysis of the BxD 22 strain was examination of the chemical components of collagen. The chemical analyses were preliminary, including a small number of tail tendon samples at several different ages, and only examined the BxD 22 RIs and one (DBA/2J) of the progenitor strains of the BxD RIs.

Sell & Monnier (1997) reported increased levels of pentosidine, an advanced glycated endproduct, in older animals with increased TTBT. At all ages analyzed, the levels of pentosidine were lower in both males and females in the BxD 22s than the DBA/2J parental strain. These pentosidine levels of the BxD 22s indicate that the increased TTBT is likely not due to advanced glycated endproducts.

Furthermore, the concentration of hydroxypyridinoline was lower in the BxD 22 mice than the DBA/2J mice for both sexes. Hydroxypyridinoline is a crosslink component, with higher levels expected with increased crosslinking and presumably with age. Similar to the pentosidine levels, it was expected that the levels of hydroxypyridinoline would be greater in the BxD 22s, but this was not observed.

These results suggest that the unusual phenotype of the BxD 22 RIs is not a simple function of the classically understood biochemistry of collagen. The small sample sizes are limiting, but serve to generate possible hypotheses for future research.

Accepting the criterion of relationship to longevity, the extreme break time of the BxD 22s should correlate with an early death of these animals, but this strain does not have an early mortality. Their median lifespan of 697.0 days ranks 12<sup>th</sup> of 25 among the progenitor and BxD RI strains in this study. Though the TTBT values of this strain do not correlate with longevity, it may be an important factor in functional aging of the animals, with the stiffer collagen potentially affecting activity or mobility of the animals.

### **Future Directions for BxD 22 RI strain investigations**

To further the understanding of the genetic influence of the extreme TTBT of the BxD 22 RI strain, a more complete Mendelian design should be undertaken, (reciprocal  $F_1$ s, two backcross generations and an  $F_2$ ) with both the C57BL/6J and DBA/2J progenitor strains with the BxD 22 RI strain.

Future directions to understand the chemical components of the crosslinking in the BxD 22 RI strain would be to examine the other BxD RI strains allowing for comparisons across all animals. Furthermore, these samples should assess animals across the lifespan, in order to assess age-related changes of the values of total collagen content in addition to crosslinking components such as hydroxypridinoline and advanced glycated endproducts including pentosidine.



## Chapter 6

### QTL Analysis of Tail Tendon Break Time

Preliminary broad-sense heritability estimates in this study indicate a moderate genetic influence on TTBT. For the BxD RI mice, the heritability estimates for the males and females respectively are 0.35 and 0.55 at 200 days of age, 0.38 and 0.69 at 500 days of age, and 0.47 and 0.35 at 800 days of age.

These estimates provide the basis for further examination of the genetic influences on TTBT, including the possibility of a QTL analysis to identify the genes influencing TTBT.

### Statistical Analyses

Interval mapping in the B6D2F<sub>2s</sub> was conducted on raw data, utilizing sex as a covariate. Adjustments were made to the BxD RI data to correct for sex differences by subtracting the difference between the male and female mean within strain from each individual male measurement, prior to interval mapping. Animals with scores exceeding four standard deviations from the overall group mean were removed as outliers for both the B6D2F<sub>2s</sub> and the BxD RIs. In addition, the BxD 22 strain was excluded from the analyses as an extreme outlier. Early mortality loss eliminated the BxD 13 strain from the analyses of the 500 and 800 day old mice. Furthermore, the analyses of the 800 day

BxD RI mice did not include the BxD 8, 14, 16, and 33 strains because fewer than 4 animals survived to 800 days of age for these strains.

## Results

### QTL Analysis of TTBT in B6D2F<sub>2</sub> intercross

Linkage analysis was used to associate microsatellite marker genotypes with TTBT. Suggestive or significant QTLs influencing TTBT at 200, 500 and 800 days of age were identified on chromosomes 2, 3, 4, 5, 6, 10, 15, and 17 (Table 6-1).

At 200 days of age, the QTL on chromosome 10 was considered significant, with the remaining QTLs on chromosomes 2, 3, 5, and 17 identified as suggestive. The D2 alleles of the QTLs on chromosomes 2, 3, and 17 were associated with increased TTBT, and on chromosomes 5 and 10 with decreased TTBT. At 500 days of age, two suggestive QTLs were identified on chromosomes 6 and 15. The B6 allele of the QTL on chromosome 6 was associated with increased TTBT, and on chromosome 15 with decreased TTBT. Data from 800 days of age identified one suggestive QTL located on chromosome 4 with the D2 allele on the QTL associated with increased TTBT.

Table 6-1: Multiple-regression model for TTBT at different ages of B6D2F<sub>2</sub> mice

Age	Chr	Peak Marker	Source	1.5-LOD, cM	%Variance	P Value	Inc. Allele/Allelic Model*
200			Sex		8.5	1.01e-06	
	2	D2Mit30	Chr 2 at 46 cM	7.0 – 109.0	2.1	0.011069	D2/♀ H ♂ A
	3	D3Mit42	Chr 3 at 55 cM	34.4 – 64.4	3.4	0.000604	D2/H
	5	D5Mit227	Chr 5 at 15 cM	9.0 – 39.0	4.2	0.001131	♀ B6 ♂ D2/♀ A ♂ R
			Chr 5 at 15 cM : sex		2.1	0.009521	
	10	D10Mit36	Chr 10 at 29 cM	22.0 – 38.0	5.3	0.000135	♂B6 ♀ D2/♀ H ♂ D
Chr 10 at 29 cM : sex				4.6	4.98e-05		
17	D17Mit93	Chr 17 at 46 cM	26.2 – 56.7	2.4	0.005499	D2/A	
		Model		19.3			
500			Sex		6.8	0.00976	
	6	D6Mit111	Chr 6 at 64 cM	48.5 – 70.5	5.2	3.21e-05	B6/A
	15	D15Mit105	Chr 15 at 36 cM	17.8 – 52.8	3.2	0.00161	D2/D
			Model		10.3		
800	4	D4Mit190	Chr 4 at 79 cM	3.9 – 79.0	7.1	5.292718e-05	D2/R
			Model		7.1		

Chr, chromosome; 1.5-LOD, cM, support interval of 1.5-LOD drop in cM determined from interval mapping; Inc Allele, Increasing Allele; A, additive; D dominant (dominant effect of the allele associated with an increase in TTBT); H, heterosis; R, recessive (recessive effect of the allele associated with an increase in TTBT); \*Listed as combined sexes unless different for sex as noted.

Figure 6-1 and Figure 6-2 show the modes of gene action of the D2 and B6 alleles for males and females at the peak marker for each QTL identified, allowing potential differences between sexes to be revealed. At 200 days of age, there were sex differences in the allelic effects of the marker near the QTL peak on chromosome 2. For males, an additive effect was apparent, with the mean TTBT value of the BD heterozygotes intermediate between the D2 (high TTBT) and B6 (low TTBT). In females, the BD heterozygotes had a mean TTBT that was higher than either the D2 or B6 homozygotes, indicating slight heterosis. Both males and females displayed heterosis for the QTL on chromosome 3, while on chromosome 5 a significant sex interaction was shown. The males exhibited slight heterosis, while the allelic effect for females was additive, with the

BD heterozygote TTBT value intermediate to that of the B6 homozygotes and D2 homozygotes. The QTL on chromosome 10, like the one on chromosome 5, showed significant interactions with sex, displaying dominance in the males (B6 homozygote and BD heterozygote TTBT values higher than D2 homozygote), and heterosis in the females. The QTL on chromosome 17 exhibited an additive effect for both males and females.

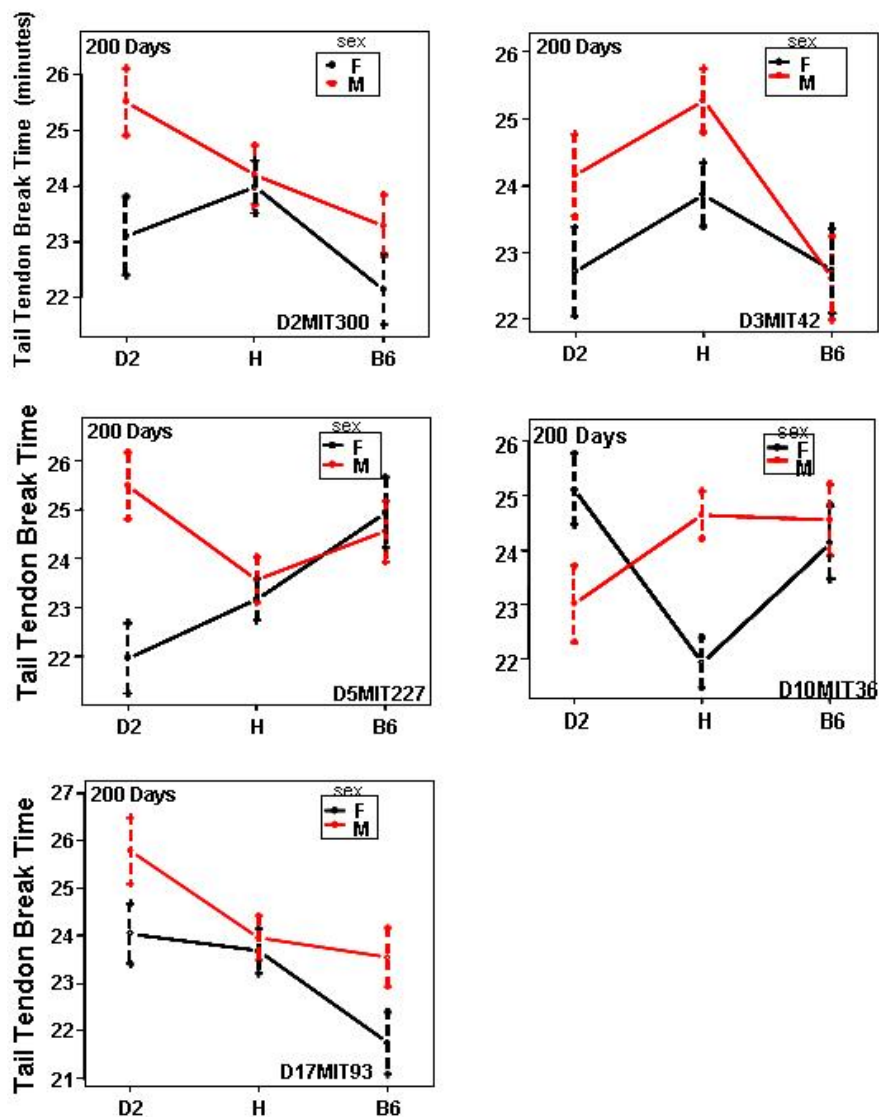


Figure 6-1: Allelic effects of the markers closest to the quantitative trait loci (QTL) peak in the B6D2F<sub>2</sub> intercross at 200 days. Values are means  $\pm$  SE for males and females separately. B6, homozygous for C57BL/6J; H, heterozygous, D2, homozygous for DBA/2J.

At 500 days of age, an additive effect was present for the QTL on chromosome 6 for both males and females, with the B6 homozygotes having the highest mean TTBT value. The D2 allele exhibited some degree of dominance for the QTL on chromosome 15 for both males and females.

At 800 days of age, the QTL on chromosome 4 showed an additive effect for both males and females, with the D2 homozygotes having the highest mean TTBT value. The characteristics of the QTLs including 1.5 LOD confidence intervals, variance attributed to each QTL, p value, and allelic model for the three age groups are summarized in Table **6-1**.

The multiple regression models used in the R/qtl program included sex as a covariate for each analysis, with only two QTLs nominated having significant interactions with sex. The effect of the QTL on chromosome 2 at 200 days of age was retained in the regression model at the  $p < 0.05$  level of statistical significance, whereas the effect of the QTL on chromosome 17 was significant at the  $p < 0.01$  level.

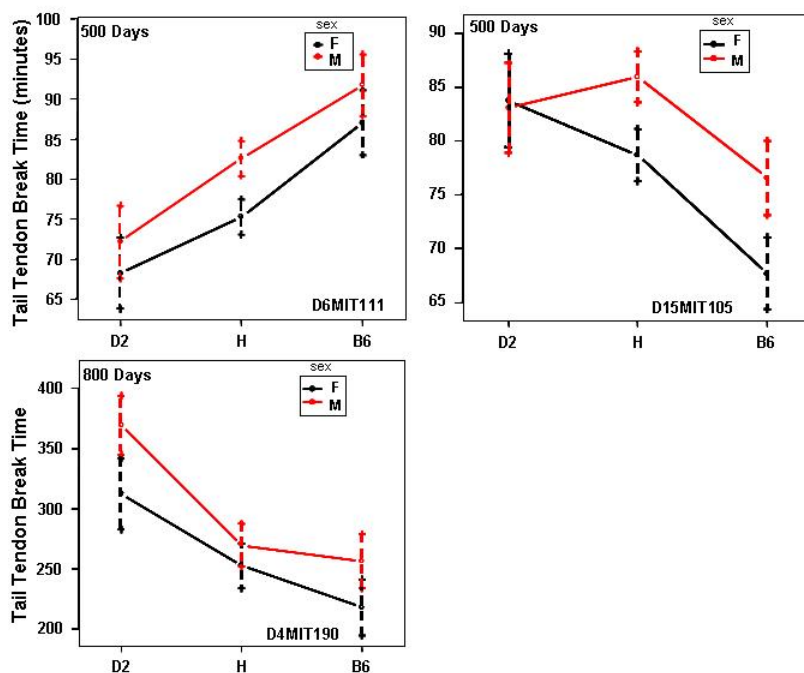


Figure 6-2: Allelic effects of the markers closest to the quantitative trait loci (QTL) peak in the B6D2F<sub>2</sub> intercross at 500 and 800 days. Values are means  $\pm$  SE for males and females separately. B6, homozygous for C57BL/6J; H, heterozygous, D2, homozygous for DBA/2J.

The effect of the QTL on chromosome 3 was retained in the regression model at the  $p < 0.001$  level. The QTLs on chromosome 5 & 10 both had significant sex-by-QTL interactions in addition to the effect of the QTLs. The effects of the QTL and the sex-by-QTL interaction on chromosome 5 were significant at the  $p < 0.01$  level, whereas on chromosome 10 the effects of the QTL and the sex-by-QTL interaction were retained in the model at the  $p < 0.001$  statistical significant level.

At 500 days of age the QTL on chromosome 6 and the QTL on chromosome 10 were retained in the regression model at  $p < 0.01$  level of statistical significance, whereas

the QTL on chromosome 4 at 800 days of age was retained in the model at  $p < 0.001$  level of statistical significance.

It is notable that the identified QTLs are different in each age group. Figure 6-3 displays an overlapping view of the three ages illustrating changes of the QTLs over time from young adult mice to elderly mice.

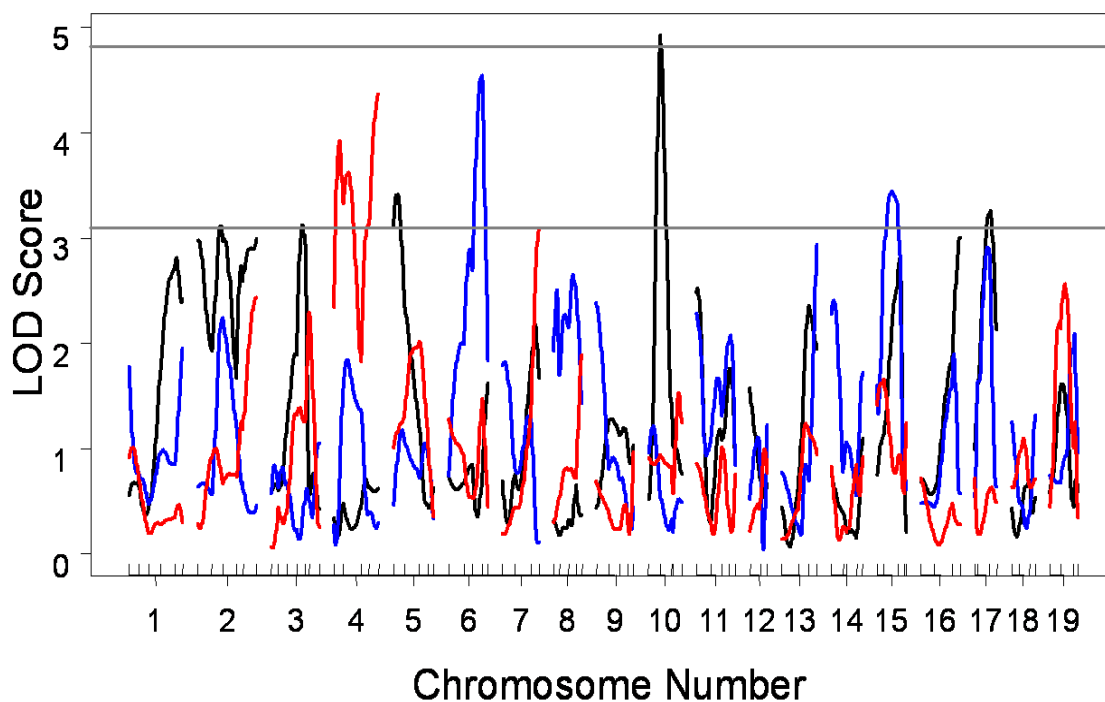


Figure 6-3: LOD plots of 200, 500 & 800 day old B6D2F2 intercross. — Black line, 200 days of age, — blue line, 500 days of age, — red line, 800 days of age. Gray lines represent suggestive (LOD = 3.1) and significant (LOD = 4.8) thresholds.

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*QTL verification testing and linkage analysis in BxD RI strains*

For each QTL nominated in the B6D2F<sub>2</sub> analyses, the 1.5 LOD drop-off interval was calculated (Table 6-1), followed by identification of the markers in the BxD RIs that fell within these support intervals. Analysis of variance was performed on single markers, using TTBT strain means. Furthermore, the statistically significant relationships within each interval were corrected for multiple comparisons (i.e., the number of markers within the interval in the BxD RI database) by the Bonferroni method (Lionikas et al., 2005). A one-tailed test was used as a conservative method for multiple comparisons, because comparisons are not completely independent due to linkage of microsatellite markers. Following the adjustment for multiple comparisons, the effect on chromosome 4 retained statistical significance, providing replication of the QTL at 800 days of age from the B6D2F<sub>2</sub> intercross results in the BxD RI mice. The effects of the QTLs at 200 and 500 days of age did not attain statistical significance in the RIs after the adjustment for multiple comparisons. The five QTLs nominated in the B6D2F<sub>2</sub> data at 200 days of age, and the two QTLs nominated at 500 days of age were not confirmed in the BxD RIs.

Interval mapping was also used to search the RI data for QTLs other than those nominated in the B6D2F<sub>2</sub> animals. One QTL at 800 days of age on chromosome 1 was identified in a combined analysis of males and females. This QTL, positioned at 59.7 cM, had a peak LOD score of 3.40, with the 1.5 LOD drop-off interval from 54.2 – 62.2 cM. The D2 allele contributed to increased TTBT for this QTL.

### *Candidate Genes*

Candidate genes were sought within the QTL regions through first determining the genetic map location of the peak marker locus. Using the 1.5 LOD drop interval, candidate genes were identified using Mouse Genome Informatics through Jackson Laboratories (Jackson Laboratories, 2007). To determine further whether the identified genes were pertinent for this analysis, the physical map location of the peak marker locus was identified, and the Perlegen mouse SNP database was used to determine the extent of haplotype differences between B6 and D2 strains. The Perlegen database was constructed through resequencing the nuclear DNA genomes of 15 inbred laboratory mouse strains, using the publicly-available sequence of strain C57BL/6J as a standard. With this approach, candidate genes residing within regions of haplotype similarity (no polymorphism present) may be excluded, and SNPs of potential functional importance in candidate genes in the regions of haplotype diversity may be identified.

### **Discussion**

Mouse tail tendon fiber denaturation in urea measured as tail tendon break time has long been used as a biomarker of aging, though the specific underlying genetic influences for inter-strain variation have not yet been identified. The results of this study are consistent with a robust genetic influence, though the effects are age-dependent. In the F<sub>2</sub>S, different QTLs are nominated at each age group. The QTLs identified at 200 days exhibited a greater influence on TTBT than the QTLs at 500 days of age, with the

least influence at 800 days of age. In the BxD RIs, the heritability declines with age, though only one QTL was identified: at 200 days of age.

The total phenotypic variance accounted for by the QTLs and covariates in the B6D2F<sub>2</sub>S was 19.3, 10.3 and 7.1% for 200, 500 and 800 days respectively, with much of the phenotypic variance attributable to gender. Furthermore, the effects of the QTLs account for a small proportion of the estimated heritabilities for each age group. These results indicate that many genes may contribute to TTBT, presumably many genes with small effect size contributing to the total variation, and that environmental influences have a great impact on TTBT that increases with age.

The results presented here are in agreement with previous studies documenting an increase with age in mean TTBT for each strain, with the shorter-lived DBA/2 mice exhibiting higher break times than the C57BL/6 animals (Higgins et al., 1991). Sell and Monnier, (1997) reported TTBT values and tissue pentosidine (an advanced glycated endproduct) concentrations increasing with age in DBA/2 and C57BL/6 mice, with the rate of increase higher in DBA/2 mice. They also found that dietary restriction significantly inhibited the age-related increase of both TTBT and pentosidine formation in DBA/2 mice, but only affected TTBT in C57BL/6 mice. Furthermore, dietary restriction also had differential effects on lysyl oxidase-mediated crosslinking and non-enzymatic glycation of collagen (Reiser, 1994). Though lysyl oxidase-mediated crosslinking and non-enzymatic glycation increased with age, non-enzymatic glycation of collagen was attenuated by dietary restriction whereas lysyl oxidase-mediated crosslinking was not. These data suggest that non-enzymatic glycation may be an

important variable affecting TTBT. Genetic variation in the generation and metabolism of advanced glycation end-products may therefore influence the TTBT.

Most QTLs identified in this study were nominated in the F<sub>2</sub> population and not confirmed in the RI mouse strains, with the only confirmation at 800 days of age. The small sample size of the BxD RIs (n=23 strain means used) limits the power to detect QTLs in the analyses (Churchill & Doerge, 1994). As a result, QTLs with small effects may be undetectable in the BxD RI mouse population. However, lack of verification is not conclusive evidence that the QTLs do not exist: they offer suggestive hypotheses for further research such as genotypic selective breeding and further investigations of candidate genes. Therefore all QTLs nominated were examined further.

Gender also influenced the action of QTLs. The regression model indicated a sex-by-QTL interaction at 200 days for QTLs on chromosomes 5 and 10. Examination of allelic effects (Figure 6-1) for the QTL on chromosome 5 revealed differences for males and females with females exhibiting an additive effect and males expressing heterosis in the direction of shorter TTBT. Inspection of the allelic effect for the QTL on chromosome 10 presents significantly different effects for the males and females, with males showing dominance, while the females display heterosis in a decreasing direction. These are the only QTLs noted in the regression and allelic effects models for the three age groups that confirm significant sex differences. The mechanisms underlying these sex-specific effects are unknown. Interactions of mitochondrial or sex-linked genes, or hormonal influence of the polymorphic autosomal genes, are obvious possible factors.

### *Candidate Genes*

TTBT largely reflects the ability of the tendon structure to withstand tensile force. This ability is primarily due to the presence of collagen fibrils in the extra-cellular matrix of the tail tendon that have a large diameter and are arranged in parallel arrays. Tendon fibrils are predominantly, though not exclusively, comprised of Type I collagen (McBride et al., 1997). For example, Type VI collagen is present during mouse tail development as well as among thick collagen fibrils in the adult tendon (Watanabe M, et al, 1997). Type XI collagen is also expressed in the tail during mouse development. How the long parallel collagen fibrils are deposited into the extra-cellular matrix has not been completely defined, although a variety of molecules may influence tail tendon structure (Canty et al., 2004). Growth Differentiation Factor 5 (GDF-5) deficiency increased the proportion of medium diameter collagen fibrils in tail tendon (Clark et al., 2001).

The search for candidate genes contributing to QTL effects (Jackson Laboratories, 2007; NCBI Entrez Gene, 2007) was restricted to those whose function was already established as related to TTBT physiology, were within the 1.5-LOD support interval of the QTLs identified, and were in a region of haplotype diversity between C57BL/6 and DBA/2 strains. Candidate genes were identified in two of the eight support intervals for the QTLs nominated. The *Coll1a1* gene located on chromosome 3 was situated within the identified 1.5-LOD support interval, and codes for an alpha subunit of Type XI collagen. Type XI collagen is expressed in the tail during mouse development, and controls matrix assembly in the ECM (Li et al., 1995; Iyama et al., 2001; Canty et al., 2004). The region of the support interval of the QTL on chromosome 4 contains three

candidate genes: *Col8a2*, *Col9a2*, and *Ddost*. *Col8a2* and *Col9a2* are both alpha subunits of Type VIII and Type IX collagens respectively. The function of the Type VIII alpha 2 subunit entails interactions with proteins in the ECM (Sutmuller et al., 1997), while the Type IX alpha 2 subunit is involved at the attachment site of bone and tendon (Fukuta et al., 1998). The tail contains coccygeal (caudal) vertebrae (Shinohara, 1999) demonstrating collagen attaches to the bone and ECM. Type IX collagen from the attachment site may be intertwined with the other tail tendon components, resulting in expression of the Type IX collagen gene in the analyses. The dolichyl-di-phosphooligosaccharide-protein glycotransferase gene, *Ddost*, located on chromosome 4, is located near the peak of the chromosome 4 QTL. It is a receptor for advanced glycation endproducts (AGEs) (Thornalley, 1998). Given that AGEs have been reported to accumulate with age (Reiser, 1994; Sell & Monnier, 1997), the *Ddost* gene in the QTL identified at 800 days appears to be a plausible QTL.

It was hypothesized that the gene for Type I collagen would appear as an underlying gene, as this is the primary type of collagen in tendons. However, the results did not nominate or confirm a QTL in the regions of Type I collagen genes (alpha 1: Chr 11, 56.0 cM; alpha 2: Chr 6, 0.68 cM) (Jackson Laboratories, 2007), indicating either that C57BL/6J and DBA/2J strains are not polymorphic for this gene, or that it does not affect TTBT. No polymorphisms exist in the *Colla1* gene, though three SNPs were identified in the *Colla2* gene: one in an untranslated region of the gene, one with the coding unknown, and the last in a coding intronic region of the gene (NCBI Entrez SNP, 2007).

### *Developmental Change*

The change of detected QTLs from 200 to 500 to 800 days of age indicates developmental change. None of the QTLs present at 200 days influenced TTBT at 500 or 800 days, similarly the QTLs identified at 500 days did not influence TTBT at 800 days of age. These findings suggest that the QTLs and underlying genes influencing TTBT at 200 days of age may be “turned off” or are not detectable at 500 or 800 days, whereas the QTLs and underlying genes at 500 days may not have been “turned on” or did not have a detectable effect at 200 days. Between 200 and 500 days of age, the QTLs identified at 500 days will presumably increase influence on the trait, while the effect of the QTLs at 200 days of age declines. At 800 days, the influence of the QTLs detected at 500 days may have decreased, resulting in non-significant or absent QTLs, while a new QTL was introduced, which was not detectable at earlier ages.

### **Conclusions**

The results partially supported our hypothesis that we would identify QTLs that influence TTBT. QTLs were nominated, though confirmation did not occur for most cases. Developmental change was suggested with different factors influencing TTBT at 200, 500 and 800 days of age.

## Chapter 7

### Overall Discussion and Conclusions

Several overall conclusions emerged from the results of all of the analyses performed on tail tendon break time in both the Biomarkers of Aging in Mice and the QTL Analysis of Age-Related Phenotypes projects.

The values of TTBT display a non-linear increase with age: changes that are systematic and robust. Mice of different genetic backgrounds demonstrate differing rates of change, though all values increase significantly during the lifespan.

TTBT at early ages does not reliably predict TTBT values later in life. Such instability may be interpreted as reflections of individual differences in rates of change across the lifespan of the animal. Not only is there an absence of consistent relationships of TTBT with itself, but also with other biomarkers. TTBT correlates with a small subset of variables, and these change with age and differ between sexes, implying dynamic underlying developmental processes.

Data from both studies show that TTBT does not reliably predict longevity. In the oldest age females in the BAM study, but no other sex or age group, TTBT correlates ( $p < 0.05$ ) with age of death. The longevity data from RI strains from the QAARP study does not show any correlation with TTBT values of the QTL sample.

Tail tendon break time is thus not a simple biomarker of aging, if prediction of longevity is deemed to be a necessary attribute. Though not clearly defined by the results of this study, TTBT may, however, be a measure of functional aging, representing a



relatively independent clock in Comfort's (1979) hierarchical clock system as a paradigm of aging.

The heritability estimates calculated in the BAM study for parent-offspring regressions and sibling analysis were very low throughout the entire lifespan. These results were inconsistent with the QAARP study, where higher heritability estimates were computed.

These differing results demonstrate that extreme complexity exists in the genetic influence of TTBT. This discrepancy of heritabilities between the two studies might be due to Gene x Environment (GxE) and Gene x Gene (GxG) interactions, that may be differentially present in the highly heterogeneous gene pool of the BAM study, in comparison to the homogeneous inbred strains of mice (BxD RI mice derived from a limited pool of allelic variability) in the QAARP project.

The results of a QTL analysis can be used for hypothesis generation for subsequent research such as candidate gene searches or genotypic selective breeding. Plausible candidate genes were identified in two of the QTL regions nominated from the completed analyses indicating the integrity of the approach. The remaining six QTLs revealed in the analyses, where no candidate genes were obvious, represent opportunities, and can be used to motivate and inform further work that may identify previously undescribed genetic factors affecting the extracellular matrix. The selective genotypic breeding procedure using the nominated QTLs would be able to create divergent lines of animals with directional distribution of increasing and decreasing genotypes for the QTLs. These lines could constitute powerful research tools for examination of the age-related attributes of TTBT.

Overall, tail tendon break time may be seen as a marker of aging. Its complexity and the lack of specificity of relationships with other aging biomarkers are underscored by the results of this study.

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- 2006                    **Course Instructor;** BBH 101: Introduction to Biobehavioral  
Health: Department of Biobehavioral Health; Summer Session #2
- 2005                    **Graduate Teaching Assistant:** BBH 101: Introduction to  
Biobehavioral Health; Department of Biobehavioral Health; Spring
- 2004                    **Graduate Teaching Assistant:** BBH 410; Developmental &  
Health Genetics; Spring; BBH 101: Introduction to Biobehavioral  
Health; Fall; Department of Biobehavioral Health

#### **Selected Manuscripts in Preparation**

**Sloane, L.B.**, Stout, J.T., Vandenberg D.J., Vogler G.P., Gerhard G., McClearn G.E. (In preparation). QTL Analysis of Tail Tendon Break Time in Mice of C57BL/6J and DBA/2J lineage.

**Sloane, L.B.**, Kovanen, V., Stout, J.T., McClearn G.E. (In preparation). Collagen with Unique Properties: Examination of Age of Appearance, Genetic Basis and Chemical Components.