APPROVAL

Name: Kristen Fay Gorman
Degree: Doctor of Philosophy

Title of Thesis:
The ‘curveback’ guppy as a model for human idiopathic-type spinal curvature

Examining Committee:
Chair: Dr. B. Roitberg, Professor

Dr. F. Breden, Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. S. Tredwell, Professor Emeritus
Department of Orthopaedics, The University of British Columbia

Dr. R. Devlin, Research Scientist
Centre for Aquatic Biotechnology Regulatory Research,
Fisheries and Oceans Canada

Dr. J. Christians, Assistant Professor
Department of Biological Sciences, S.F.U.

Dr. B. Crespi, Professor
Department of Biological Sciences, S.F.U.
Public Examiner

Dr. J. Hall, Professor Emerita
Department of Pediatrics, The University of British Columbia
External Examiner

10 September 2009
Date Approved
Declaration of Partial Copyright Licence

The author, whose copyright is declared on the title page of this work, has granted to Simon Fraser University the right to lend this thesis, project or extended essay to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users.

The author has further granted permission to Simon Fraser University to keep or make a digital copy for use in its circulating collection (currently available to the public at the Branches & Collections’ “Institutional Repository” link of the SFU Library website www.lib.sfu.ca), and, without changing the content, to translate the thesis/project or extended essays, if technically possible, to any medium or format for the purpose of preservation of the digital work.

The author has further agreed that permission for multiple copying of this work for scholarly purposes may be granted by either the author or the Dean of Graduate Studies.

It is understood that copying or publication of this work for financial gain shall not be allowed without the author’s written permission.

Permission for public performance, or limited permission for private scholarly use, of any multimedia materials forming part of this work, may have been granted by the author. This information may be found on the separately catalogued multimedia material and in the signed Partial Copyright Licence.

While licensing SFU to permit the above uses, the author retains copyright in the thesis, project or extended essays, including the right to change the work for subsequent purposes, including editing and publishing the work in whole or in part, and licensing other parties, as the author may desire.

The original Partial Copyright Licence attesting to these terms, and signed by this author, may be found in the original bound copy of this work, retained in the Simon Fraser University Archive.

Simon Fraser University Library
Burnaby, BC, Canada

Revised: Spring 2009
STATEMENT OF ETHICS APPROVAL

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

(a) Human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

(b) Advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

(c) as a co-investigator, in a research project approved in advance,

or

(d) as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Bennett Library
Simon Fraser University
Burnaby, BC, Canada
ABSTRACT

Idiopathic (meaning no known cause) scoliosis accounts for 80% of all cases of human spinal curvature, affecting an average of 3%-4% of the global paediatric population. Phenotypic variability, curve pathogenesis that coincides with growth, and the lack of an animal model that does not rely on induced curvature, have made it difficult to discover the etiopathogenesis of this complex deformity. Although a genetic basis is accepted, the specific genes, the mode of inheritance, and the proportion of phenotypic variation due to genetics are not known. Furthermore, factors that explain a propensity for curve severity have not been determined. The discovery of causative and progressive factors would be greatly facilitated by a genetic animal model. With complex human syndromes that involve interactions among genetic, physiological, and environmental forces, an important experimental approach is to first identify genes and biochemical factors in a model animal with a similar phenotype. The curveback mutant guppy [Poecilia reticulata] is the first animal model with heritable morphological and developmental similarities to human idiopathic scoliosis. This thesis describes construction of the curveback lineage and characterization of the curveback phenotype so that it can be applied as model for the discovery and exploration of biological processes that contribute to idiopathic spinal curvature. In addition, I describe our initial efforts for mapping genes that are associated with the curveback deformity. Identification of genes associated with idiopathic-type
scoliosis in curveback could help to identify biological processes that are involved in the human deformity, which would lead to methods of effective screening for early curve detection as well as possible therapeutics.

Keywords: idiopathic scoliosis, spinal curvature, animal model, guppy, teleost, genetic, vertebrae, deformity
Dedicated my mother and father, who are also my heroes, as well as two of my favourite people:

Kathleen Fay Gorman and Richard J Gorman.
ACKNOWLEDGEMENTS

The work in this thesis is the product of an exceptional collaboration between my senior supervisor, Felix Breden and myself. I appreciate that Dr. Breden has been an insightful and attentive mentor while also allowing me to independently explore many interesting research questions. I am thankful for the time that Dr. Breden has invested into my career and believe that I will be a better scientist as a result. I have tremendous respect for my supervisory committee: Dr. Robert Devlin, Dr. Julian Christians and Dr. Stephen Tredwell, as well as my examiners Dr. Judith Hall and Dr. Bernie Crespie. Not only did I enjoy their company, I also appreciate their input as well as the time committed to my thesis. Dr. Tredwell and Angie Perdios have been incredibly supportive and helpful with this work. I also am thankful for the opportunity to exchange ideas with Dr. Christopher Riley, the FABstar group, and Dr. Jaimie Scott who has been very kind and supportive over these years.

I have had the opportunity to be a part of a great lab, which has been like a second family. Corey Watson, Scott Pavey, Dr. Heather Alexander, Dr. Laura Weir, Jennifer Parent, Roozbeh Amahadi and Ben Sandkam are exceptional people who have made my working environment a pleasure. The Breden Lab gets the work done. I have had some wonderful undergraduates work with me as independent study coursework, and I am very happy to have had the pleasure to know them, and I wish them all the best in their careers.
TABLE OF CONTENTS

Approval......................................................................................................................... ii
Abstract......................................................................................................................... iii
Dedication....................................................................................................................... v
Acknowledgements........................................................................................................ vi
Table of Contents........................................................................................................ vii
List of Figures................................................................................................................ x
List of Tables................................................................................................................ xi
Glossary.......................................................................................................................... xi

Chapter 1: Introduction................................................................................................. 1
  1.1 Human idiopathic scoliosis ...................................................................................... 2
     Possible aetiologies for IS ....................................................................................... 8
     Models for the study of idiopathic-type curvature ................................................ 9
  1.2 Construction of the curveback lineage ................................................................. 10
     Selection strategy ................................................................................................. 11
     Response to selection ............................................................................................ 12
  1.4 Curveback as a model for human idiopathic-type scoliosis ................................ 13
  1.4 Thesis Overview and Author Contributions ....................................................... 15
  1.5 Literature Cited...................................................................................................... 19

Chapter 2: Teleosts as models for human vertebral stability and deformity*................. 32
  2.1 Abstract ................................................................................................................. 33
  2.2 Introduction ............................................................................................................ 34
  2.2 Spinal curvature in humans................................................................................... 35
  2.3 Idiopathic scoliosis ............................................................................................... 36
  2.4 Animal models for human idiopathic scoliosis ................................................... 40
  2.5 Spinal curvature in model teleosts ....................................................................... 42
  2.6 curveback as a model for human familial/idiopathic scoliosis
     deformity ............................................................................................................... 47
  2.7 Conclusion ............................................................................................................. 49
  2.8 Acknowledgments ................................................................................................. 50
  2.9 Literature Cited...................................................................................................... 51
LIST OF FIGURES

Figure 1.1: The original curveback cross ......................................................... 27
Figure 1.2: The curveback pedigree ................................................................. 28
Figure 1.3: The curveback phenotype .............................................................. 29
Figure 1.4: Phenotypic response to selection for severe curve magnitude ................................................................. 30
Figure 2.1: The curveback phenotype .............................................................. 65
Figure 2.2: Causes of Spinal Deformity in Fish ............................................... 66
Figure 2.3: A summary of the research conducted on model teleosts with regard to heritable spinal curvature ................................................................. 67
Figure 3.1: The curveback phenotype .............................................................. 88
Figure 3.2: Qualitative classification for curve types ......................................... 89
Figure 3.3: Anterior curve ratio ....................................................................... 90
Figure 3.4: Computed tomography scan of curve apex .................................... 91
Figure 3.5: Gender distribution for qualitative scores ....................................... 92
Figure 3.6: Curveback pathogenesis ................................................................ 93
Figure 3.7: Complex inheritance for curve magnitude ...................................... 94
Figure 4.1: ................................................................................................. 114
Figure 5.1: The curveback phenotype .............................................................. 140
Figure 5.2: The Caudal Skeleton ................................................................. 141
Figure 5.3: Normal vertebral and intervertebral microanatomy of the guppy, Poecilia reticulata ................................................................. 142
Figure 5.4: Vertebral shape distortion associated with curvature .................. 144
Figure 5.5: Vertebrae significantly affected by curvature ............................... 145
Figure 5.6: Vertebral and intervertebral defects associated with spinal curvature in the curveback guppy ................................................................. 146
Figure 6.1: Distribution of Curve Magnitude within Study Population .......... 166
Figure 6.2: Phenotype measured .................................................................. 167
Figure 6.3: Component Measures ................................................................ 168
Figure 6.4: Allometry between precaudal and caudal lengths ..................... 169
Figure 6.5: Caudal portion increases with curve magnitude .......................... 170
Figure 6.6: Apparent growth abnormality ...................................................... 171
Figure 7.1: Marker 1 on linkage group 'a' ....................................................... 189
Figure 7.2: Marker 2 on linkage group 'b' ....................................................... 190
Figure 7.3: Least Squares Means Plot ............................................................ 191
LIST OF TABLES

Table 1.1: Summary of human genetic studies after 1990................................. 31
Table 2.1: Types of progressive/structural spinal deformities in humans*        
and reported occurrence in teleosts....................................................... 68
Table 2.2: ...................................................................................................... 69
Table 3.1: Parallels between human and guppy idiopathic spinal curvature ................................................................. 95

Table 5.1: Five ratios used to characterize vertebral distortion....................... 148
Table 5.2: Vertebrae with significant values from ratio tests.......................... 149
Table 6.1: Comparison of length measurements in curved versus non-        
curved fish................................................................................................. 172
Table 7.1: Distribution of Phenotypes in mapping crosses ......................... 192
Table 7.2: Marker coverage for Each Linkage Group .................................. 193
Table 7.3: Results from Interval mapping..................................................... 194
# GLOSSARY

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic-type spinal curvature</td>
<td>Spinal curvature with no apparent fusions or breaks, and no apparent cause. Occurs in otherwise healthy animals. In all cases, curvature develops during growth.</td>
</tr>
<tr>
<td>Genetic scoliosis</td>
<td>A structural spinal curve that is inherited according to a known genetic pattern.</td>
</tr>
<tr>
<td>Etiopathogenesis</td>
<td>Causative factors that explain the development of a deformity or disease.</td>
</tr>
<tr>
<td>Penetrance</td>
<td>The proportion of individuals in a population having a mutation that causes a particular disorder who display the phenotype associated with that disorder.</td>
</tr>
<tr>
<td>Expressivity</td>
<td>The extent to which an inherited trait is manifested in an individual.</td>
</tr>
<tr>
<td>Cobb’s angle</td>
<td>A measurement used for the evaluation of the magnitude of scoliotic curves. Anterior/posterior radiographs are used to identify the upper and lower end vertebrae of a curve (the most superior and inferior vertebrae that are least displaced or rotated and have the maximally tilted end plate). Lines are drawn along the superior end plate of the superior vertebra and along the inferior end plate of the inferior vertebra. The angle at the intersection of these two lines is the Cobb’s angle.</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
1.1 Human idiopathic scoliosis

Definition

Human idiopathic scoliosis (IS) is a type of spinal curvature that has no apparent aetiology and occurs during growth in otherwise healthy children. The deformity is characterized by a lateral curvature of the spine, but often occupies all three planes of the human body in the form of sagittal (anterior/posterior) and coronal (left/right) deviation of the spine and axial rotation of the vertebrae (occupying the horizontal plane). Thus, idiopathic scoliosis can be considered a four-dimensional deformity, because progression of the three-dimensional deformity occurs during growth (through time).

The IS phenotype is further characterized by variability for: curve morphology and magnitude, the age of curve onset, the rate of curve progression, and the propensity for curvature to progress or stabilize or resolve. Variability for age of onset has been partitioned into three general categories that are supposed to coincide with the three major phases of increased growth velocity during childhood and adolescence: infantile (0-3 years), juvenile (4-9 years), and adolescent (10 years to maturity). Some researchers propose that it is more appropriate to partition age into two categories: before 5 years and after 5 years, because children with curvature that begins prior to the age of 5 are at risk of cardiopulmonary compromise (Dickson, 2001). There is a female bias for curvature, the proportion of which increases with the magnitude considered (Weinstein, 2001). For infantile and juvenile onset IS, there is a chance that curvature will resolve to normal or nearly normal before the child reaches maturity.
(Dickson, 2001; Warner, 2001). Besides an unknown aetiology, why some curves progress while others stabilize or resolve, and how progressive factors relate to causative factors is unknown.

According to the Scoliosis Research Society, among the ten major aetiological categories that explain human spinal curvature, idiopathic scoliosis (IS) represents 80% of all observed cases (according to the Terminology Committee of the Scoliosis Research Society, 1976; OMIM 181800, Online Mendelian Inheritance in Man). The debilitating deformity affects 0.15%-10.0% of the global paediatric population (Lonstein, 1994; Axenovich, et al., 1999; Reamy, et al., 2001; Asher and Burton, 2006). The deformity imposes a substantial cost on the healthcare system. According to the National Scoliosis Foundation (www.scoliosis.org/info.php), each year scoliosis patients in the US and Canada make more than 650,000 visits to physicians, an estimated 33,000 children are braced for scoliosis, and 42,000 patients undergo spinal fusion surgery. In 2004, the estimated cost of treating patients hospitalized with a diagnosis of idiopathic scoliosis in the United States alone was over $3 billion (American Academy of Orthopedic Surgeons, 2008).

Genetics

A genetic basis for idiopathic scoliosis is generally accepted, based on familial clustering (Garland, 1934; Wynne-Davis, 1968; Riseborough and Wynne-Davis, 1973) and a high concordance among monozygotic twins (Kesling and Reinker 1997; Inoue, et al., 1998). However, the mode of inheritance and the proportion of phenotypic variation due to genetics remain a matter of debate.
Difficulty in describing the inheritance of IS might be due to inconsistent pedigree construction between studies. Early studies that surveyed curvature by questionnaire or physical examination are especially prone to inconsistencies (Bashiardes, et al., 2004). Although Fisher and De George (1967) could not find sufficient evidence for simple genetic factors associated with IS, and Wynne-Davies (1968) could not distinguish between a dominant or multigenic mode of inheritance, many studies conducted in the mid 1970's report a dominant inheritance pattern. However, these studies are based on evaluation of single families or small collections of families using surveys or physical screening for curvature (Garland 1934; Gilly et al, 1963; Filho and Thompson, 1971; Riseborough and Wynne-Davies, 1973; Robin and Cohen, 1975; Wynne-Davies 1975). The sample size and composition used for analysis can effect the mode of inheritance inferred. For example, while Bell and Teebi (1995) reported autosomal dominant inheritance based on a family in which a father and 2 daughters had IS, Axenovich et al. (1999) used 101 pedigrees to suggest that inheritance was either dominant or multigenic, depending on the magnitude of the curve evaluated (pronounced curves showed a significant contribution from a major causal gene while minor curves showed no significant major gene effect).

How data is collected and evaluated can also affect perceived inheritance. For example, Cowell et al (1972) provided evidence for X-linked dominant inheritance. However, these have been disputed after re-evaluation of original X-rays for the study subjects (Riseborough and Wynne-Davies, 1973; Horton, 2002). In 2003, Justice and others reintroduced the possibility of X-linked
dominant inheritance using a different dataset, and so whether the X-chromosome is involved in IS remains unclear. Early reports on high concordance among monozygotic twin pairs are based on case reports and hospital registers. Anderson et al (2007) points out that these samples over represent concordant pairs because there is a double chance of being discovered. In contrast to early studies, recent studies report phenotypic variability (low concordance rates for curve morphology, magnitude, and prognosis) among pairs of monozygotic twins (Anderson, et al., 2007; Hermus, et al., 2007; Weiss 2007). The studies question the assumed genetic basis for IS.

More recent studies generally use radiographs to calculate a Cobb’s angle (see glossary) for diagnosis. However, radiographs are usually recommended only if a spinal deviation is noted at a clinical screening (Lowe, et al., 2000). A recent review of screening methodology showed that the most common method of curve detection was by family or friends (Fazal and Edgar, 2006). The majority of cases detected by such means presented a Cobb’s angle of such magnitude that non-operative measures are ineffective, suggesting that curves of lesser magnitude are not detected or reported (Fazal and Edgar, 2006; Goldberg, et al., 2007). It has been suggested that adolescent onset idiopathic scoliosis is present in a latent form in younger children who manifest a minor degree of curvature (Stirling, et al., 1996). Genes that correlate to curves of low magnitude that do not merit physician attention will segregate in the human population undetected and could account for the high incidence of ‘spontaneous’ IS. Thus, minor curves
have the potential to be overlooked and this would obscure the true inheritance of curvature.

Recent genetic studies attempt to link curvature to candidate genes or genomic markers through pedigree-based or population-based linkage analysis and association studies, and these have given inconsistent results (Table 1.1). With the exception of 3 studies, those that target candidate genes can be broken into two major categories: 1) genes having to do with structural aspects of spinal integrity [collagen type I & type II (Carr, et al., 1992); fibrillin-1, elastin (Miller, et al., 1996); aggrecan (Zorkol'tseva, et al., 2002, Sharipov et al, 2006, Marosy, et al., 2006); fibrillin-3 (Cao, et al., 2008); pectus excavatum (Gurnett, et al., 2009); mantrillin-1 promotor (Esposito, et al., 2009)], or 2) genes having to do with the endocrine system [vitamin D receptor, estrogen receptor, CYP17 (a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens) (Inoue, et al., 2002a and 2002b; Zhang, et al., 2009); insulin growth factor-1 (Yeung, et al., 2006); calmodulin-1 and growth hormone receptor (Zhuang, et al., 2007); melatonin receptor-1B promotor (Qiu, et al., 2007); growth hormone promotor, melatonin receptor-1A (Qiu, et al., 2008a, 2008b); tryptophan hydroxylase-1 and acrylalkylamine N-acetyltransferase, both critical enzymes for melatonin biosynthesis (Wang, et al., 2008); calmodulin receptor-1 and estrogen receptor-1 (Zhao, et al., 2008; Raggio, et al., 2009)]. The other 3 candidate genes tested are: SNTG1 (a neuronal gene associated with dystrophan, the Duchenne muscular dystrophy gene, and located within a region identified as linked to curvature based on an earlier association
study) (Bashiardes, et al., 2004); chromodomain helicase DNA binding protein 2 (modifies nucleosome binding and remodelling) (Kulkarni, et al., 2008); depeptidyl peptidase 9 (a serine exopeptidase) (Qiu, et al., 2008c). The sample sizes and results for these studies are summarized in table 1.1.

In addition to candidate gene studies, there are at least 9 genomic studies that have screened the genome of curved and non-curved individuals with human SNP (single nucleotide polymorphism) markers to determine regions that are associated with curvature (see table 1.1). Among these studies, the highest LOD score reported for significant linkage of a genetic marker to curvature is 4.93 (chromosome 19, Chan, et al., 2002), and the lowest is 1.69 (X chromosome, Justice, et al., 2003). Some studies use the same datasets (groups of individuals) to identify different loci (Justice, et al., 2003; Miller, et al., 2005; Alden, et al., 2006). These studies suggest that how strongly a marker is linked to curvature depends on the magnitude of the curve considered. Among the nine studies outlined in table 1.1, two studies show associations between IS and chromosomes 9 and 17 (Miller, et al., 2005; Ocaka, et al., 2007), and another implicates just chromosome 17 (Salehi, et al., 2002). Also, chromosome 19 has been associated with IS in two different studies (Chan, et al., 2002; Alden, et al., 2006). Chromosomes 8 (Bashiardes, et al., 2002), 6 and 16 (Miller, et al., 2005), 5 and 13 (Miller, et al., 2006), and 12 (Raggio, et al., 2009) have also been associated with idiopathic-type spinal curvature. The current view is that IS is a complex genetic disorder with multiple predisposing genes segregating in the human population, exhibiting complex genotype by environment interactions
To summarize, although it has a genetic basis, idiopathic scoliosis does not demonstrate a clear pattern of inheritance that would define it as 'genetic scoliosis'. This may be a result of inconsistent pedigree construction between studies, or that there are multiple genes for curve predisposition segregating among different populations.

Possible aetiologies for IS

Multiple hypotheses have been proposed for the aetiology of IS, but none of them are supported by convincing evidence, and many studies that investigate the hypotheses give conflicting results (reviewed in Miller, 2001). Hypotheses include: structural pathologies such as defects within the connective tissue, skeletal muscle, ligaments, vertebral bodies, or intervertebral discs; defects involving neuromotor mechanisms such as proprioception, postural equilibrium, or the cerebral cortex; defects involving growth and the endocrine system such as estrogen, melatonin, growth hormone, and/or vitamin D. In many cases, abnormalities that correlate to idiopathic scoliosis are challenged because it is unclear whether they are a cause or effect of the curvature. For example, studies that test the expression of melatonin receptor mRNA (Qiu, et al, 2007) or aggrecan gene expression (using keratan sulphate as a marker for genetic change) (Zaidman, et al., 2006) cannot rule-out that changes in gene expression are a consequence of altered biomechanical activity associated with curve pathogenesis and not a primary aetiology.
It is possible that in addition to a predisposition, there are secondary factors that interact with the primary aetiology and affect the course of curve pathogenesis (i.e. risk factors). Examples of secondary factors are: innate morphology (e.g. ectomorphy), growth rate (e.g. peak velocity in males vs. females), hormonal (e.g. growth hormone, estrogen, melatonin), behavioural (e.g. activity level, habits), and environmental differences (e.g. vitamin D, latitude), to name a few. Considering that idiopathic scoliosis has a genetic basis, and that there may be multiple pathological subgroups demonstrating a similar phenotype, I herein refer to ‘idiopathic scoliosis’ as idiopathic-type scoliosis.

Models for the study of idiopathic-type curvature

Most animal models used to study the etiopathogenesis of idiopathic-type scoliosis rely on induced curvature, and so it is controversial whether such experiments are measuring primary or secondary influences of the end phenotype (reviewed in Kawakami et al., 1999; Braun, et al., 2006; Fagan, et al., 2009). In animals with non-induced curvature, the type of scoliosis studied is congenital (e.g. hens, Ruble, et al., 2002; scoliosis mouse, Adham, et al., 2005), or occurs only in sexually mature animals (e.g. chickens, Riggins, et al., 1977; a mature Orangutan, Naique, et al., 2003), and so it is unclear how well the observed deformity relates to IS. Chapter four will explore the animal models used to study IS in detail. In order to understand the genetic basis and elucidate the aetiology of idiopathic-type scoliosis, a model with heritable curvature and a phenotype similar to human IS is necessary. The curveback guppy is the first
animal model with heritable idiopathic-type curvature and remarkable phenotypic similarities to the human IS syndrome.

1.2 Construction of the curveback lineage

Overview

The guppy, Poecilia reticulata, is a live-bearing species of teleost that has been used as a model animal for answering evolutionary and genetic questions since the 1920’s. The curveback lineage was established from laboratory guppies that are derived from a wild population caught in Central Cumaná, Venezuela and raised under standardized conditions since 2000. The curveback pedigree originated from a curved male crossed to a normal, unrelated female in 2003 (figure 1.1), followed by full-sib mating and backcrossing that has continued to present. The curveback pedigree consists over 2500 individuals and 13 generations (figure 1.2). Two thousand curveback adults have been scored for their curve phenotypes, photographed, and frozen as resources for further genetic and morphological study. The first 1000 individuals of the pedigree were scored for curvature throughout development (between 1 and 5 times per week) in order to document curve pathogenesis. Scoring curvature consists of viewing individual fish in a plastic view tank that is held up to the ambient light source. The spine is visible without magnification.

The curveback phenotype characterized in this thesis is a dorsal-ventral deviation of the spine that occurs exclusively on the sagittal plane of the fish. Within the tail, there is a primary anterior curve with the concave side dorsally
directed (lordosis), and a secondary posterior curve with its concave side ventrally directed (kyphosis) (figure 1.3a). Curve onset is after birth and curvature develops during growth and generally does not progress past sexual maturity. The final magnitude of a curve is highly variable. To quantify observed variation for curve magnitude throughout growth and among a large population, I devised a qualitative scale for the magnitude of curvature that will be described in chapters two and three and referred to throughout this thesis. In addition to variable curve magnitude, within the curveback pedigree there is variability for age of curve onset, the rate of curve progression, and the propensity for a curve to stabilize, progress in magnitude, or resolve to normal or nearly normal. In addition, there is a female prevalence for severe curves. These aspects of the phenotype are explored in chapters two and three.

Selection strategy

From the original cross that established the curveback lineage, six F1 were scored as adults and none of them had curvature, suggesting that curveback is a recessive trait. When the normal F1 progeny were crossed to one another, variable curve magnitudes were observed in the F2, demonstrating trait complexity. Furthermore, F2 mating pairs with severe curvature produced F3 offspring with various degrees of curvature, including normal, indicating, as with the human phenotype, the possibility of multiple genes or variable expressivity. The entire curveback lineage has been propagated by sibmates and backcrosses. At F3, two individuals derived from the Central Cumaná laboratory stock but not from the curveback lineage were introduced into the pedigree
because they had severe spinal curvature similar to curveback. These two females were inbred for 3 generations from another experiment involving sibmates. The descendants of these introduced females have been inbred as a sub-lineage of curveback by sibmates and backcrosses and are included in the total number of offspring for curveback.

Between 2003 and 2007, the selection regime for the curveback pedigree included crosses in which fish with similar curve magnitudes were crossed to one another, as well as continued directional selection for the greatest magnitude of curvature. The goals were: 1) to establish families that breed true for each of the different curve magnitudes observed, 2) to explore the phenotype in response to selection for the most severe curve magnitude. From 2008 until present, selection for severe curve magnitude continues, as well as inbreeding in order to create isogenic families that can be used as a resource for mapping the genetics associated with observed variation such as; resolving curvature, curve morphology, and relative tail length (discussed in chapter six).

**Response to selection**

At F10, analysis of inheritance of the curveback phenotype by a linear mixed effects model as implemented in AsReml (2) estimated the heritability of curvature as 0.63 with little maternal effect. Although females tend to develop greater magnitude curves, there is no difference between estimates of male versus female heritability. I was unable to create true-breeding families for different curve magnitudes because crosses using parents of any magnitude other than the most severe would give offspring with all curve magnitudes.
However, selection for the highest curve magnitude did create families with 95-99% of offspring exhibiting high magnitude curvature.

Furthermore, with inbreeding for curves of high magnitude, the curveback phenotype has evolved so that the severity of the highest magnitude curve has increased, and in some families curvature occurs on the coronal plane of the body (in addition to the sagittal) and the spine demonstrates axial rotation (figure 1.3b). Selection for coronal curvature has created families in which this trait is recurrent. At F10 the heritability for coronal curvature was curve=0.221. Moreover, in some families, selection for the highest curve magnitude has resulted in a different curve morphology than the original curveback phenotype (sagittal lordosis and kyphosis) (figure 1.4). All individuals with an altered morphology have been bred with siblings in order to test if the altered phenotype is genetic. The altered curve morphology occurs in approximately 1-5% of the offspring from a cross where one parent has altered curve morphology. I am currently inbreeding siblings that exhibit altered curve morphologies, including those with three-dimensional curvature, so that we have sufficient numbers of fish for future experimentation. The analyses in this thesis do not address the three-dimensional or altered curve morphologies because they emerged recently.

1.4 Curveback as a model for human idiopathic-type scoliosis

Generally, the genetic factors that maintain spinal integrity are not well understood. The importance of such an understanding is demonstrated by the fact that scoliosis research has been a high priority for the US National Institute of Health (NIH) since 2005 (see Congressional Appropriations Bill, House Report.
108-636). Furthermore, from 2007 until present, NIH has given priority to research regarding the genetics and underlying biological processes for various types of bone disease and skeletal deformities (Congressional Appropriations Bill, House Report 109-515). In this regard, the study of how idiopathic-type spinal curvature arises in the guppy model *curveback* is an important basic science pursuit that may lead to greater understanding of molecular and cellular mechanisms involved in spinal curvature, regardless of their immediate application to a specific condition in humans.

As a tool, the *curveback* model is highly significant because it will provide valuable insight into the basic biological nature of heritable idiopathic-type spinal curvature. The importance of developing new and appropriate (animal) biomedical models for complex human diseases is well recognized (reviewed in Swanson, *et al.*, 2004). Animal genetic models have proven successful for studying complex human phenotypes (see Gitler, 2009). Examples are: left ventricular hypertrophy (LVH) (Masciotra, *et al.*, 1999); blood pressure (Duong, *et al.*, 2007); cardiovascular disease (Dahme, *et al.*, 2009); alopecia areata (AA) (Siebenhaar, *et al.*, 2007); obesity (Jones and Ashrafi, 2009); Familial Hypertrophic Cardiomyopathy (Kittleson, *et al.*, 1999); and shear stressing response to atherosclerosis, where differences between strains of rats provided valuable insight into the causes of variation in the human phenotype (Ibrahim, *et al.*, 2003).

Characterization of phenotypic parallels between human and *curveback* idiopathic-type scoliosis phenotypes is important for the establishment of
*curveback* as a relevant biomedical model. Exploration of these phenotypic parallels and identification of genes involved in the *curveback* model has the potential to elucidate important biological pathways involved in idiopathic-type curvature. This research should help to identify biological pathways that are involved in the human idiopathic-type scoliosis (IS) syndrome, that will ideally lead to the identification causative genes, leading to more effective screening and early curve detection, and suggest possible therapeutic interventions.

1.4 Thesis Overview and Author Contributions

This thesis is organized so that the chapters are in order of submission for publication, with the last chapter unsubmitted. This order reflects the evolution of the project from a review of spinal curvature among teleosts (chapter 2) and preliminary data on *curveback* (chapter 3) to more detailed inquiries into the phenotypic parallels between human and guppy idiopathic-type scoliosis (chapters 4-6). Chapter 7 discusses our efforts to create mapping resources and locate major QTL for *curveback* so that we can positionally clone genes associated with curvature. Below I provide a brief summary of each chapter and the contributions of each co-author. Because chapters 2, 3, 4, 6 are already published (or are accepted), there is some redundancy throughout the thesis regarding the description of human idiopathic-type scoliosis, the description of animals models used prior to *curveback*, and the description of the *curveback* model.

Chapter 2 is a review of causes for spinal curvature among teleosts, with specific emphasis on model teleosts. I compare the causes for spinal curvature
among teleosts to the major categories for causes of spinal curvature among humans (according to the Scoliosis Research Society, 1976), and demonstrate that humans and teleosts have the same major causes for spinal curvature (except possibly for neurofibromatosis). I propose that teleosts are an untapped resource for the study of spinal deformities in humans, and describe our research with the curveback guppy as an example. This review was published in Comparative Biochemistry and Physiology C as a part of a special issue devoted to presenters from the 2005 Aquatic Models for Human Disease conference (Athens, Georgia). This paper is co-authored with my supervisor, Dr. Felix Breden. While I wrote the majority of the paper, Dr. Breden helped considerably in revisions for the final draft. Our goal was to target the audience of teleost scientists to convey the potential contribution of teleost spinal studies to humans.

Chapter 3 is an overview of the curveback phenotype, broken down into morphological, developmental, and genetic investigations. The purpose of partitioning the phenotype into these categories is to systematically test the feasibility of curveback as a model for IS because for the most part, the complexity of IS has been partitioned along these lines. Our preliminary analysis of the curveback phenotype suggested that it is the first genetic animal model for IS with morphological and developmental phenotypic parallels. This paper was published in the journal Spine. Our goal was to target the orthopaedic community and propose to them that teleosts have the potential to give valuable insights for human spinal pathologies, and that specifically, curveback is a feasible model for IS. I collected and analyzed the data and wrote the bulk of the paper. Dr. Breden
gave considerable time and effort to revisions for the final draft. Through consultation, Dr. Tredwell helped with the articulation of our ideas so that they may be presented to the orthopaedic community.

Chapter 4 is a brief paper published in the journal Medical Hypotheses. The paper is in response to a prevalent perspective that idiopathic-type scoliosis is a human specific or bipedal deformity. We discuss how the choice of animal models used to study idiopathic-type scoliosis has supported this notion. We present the hypothesis that there are common molecular pathways involved in the etiopathogenesis of the guppy and human phenotypes.

Chapter 5 characterizes the morphological changes in vertebrae that are associated with curvature in curveback. Other than humans, the guppy is the only animal that exhibits vertebral shape distortion in association with idiopathic-type spinal curvature. This feature of the human phenotype has been so important that it has been induced extensively in animal models. We use whole mount skeletal and histological analyses to investigate the structural and microarchitectural changes in vertebrae with the non-induced curveback phenotype. This paper will be submitted to the Journal of Morphology. I conceived of the project and designed the protocol for clear and stain, analyzed the shape data, and wrote 2/3 of the paper. Ge Jin and Rob Wallis did the clear and stain work as undergraduate independent study projects under my supervision. Ge Jin devised the seven measurements used to analyze vertebral shape and then measured each vertebra from digitized photos. Dr. Gregory Handrigan conducted the histology and wrote the relevant materials and
methods, and results that relate to his analysis. Dr. Breden helped with the analysis. Dr. Breden and Dr. Handrigan contributed to the revisions for the final draft.

Chapter 6 is a comparative study of allometric lengths in normal and curved adult curveback females. We found that although absolute body length does not correlate to curvature, among curved females the length of the tail is significantly longer relative to the length of the body, and this disproportion correlates with curve magnitude. We relate these findings to anthropometric studies in which disproportionate body lengths have been correlated to curvature in girls with IS. I conceived of and conducted the experiments, analysis, and wrote the bulk of the paper. Dr. Breden helped with the analysis and the revisions for the final paper. This manuscript has been reviewed and accepted for publication in the journal Spine.

Chapter 7 describes our initial QTL mapping efforts. I describe how we used interval mapping to analyze backcross progeny and identify two chromosomes that are significantly linked to curveback. The specific location of the mapped loci is not given because of our intention to patent some of the material derived from this chapter. Dr. Felix Breden and I conceived of the project, and Dr. Julian Christians and Dr. Breden helped with data analysis and revisions of the final chapter. I cultivated the crosses, photographed and phenotyped the offspring, analyzed the data, and wrote the body of the chapter. Roozbeh Amahadi and Parveen Rai extracted DNA. The DNA was genotyped by
Sequenom, Inc using markers developed by Dr. Christine Dryer and Dr. Detlef Weigel of the Max Plank Institute, Tübingen, Germany.

1.5 Literature Cited


Figure 1.1: The original curveback cross

The curveback pedigree started from a moderately curved male (right) crossed to an unrelated, normal female (left).
Each generation is shown as a new level (to F10), and numbers indicate an individual fish (not visible for F4-9 because there are too many individuals). Males are depicted as red, and females as yellow. The P generation shows the original cross and an additional male and female who are also derived from the Cumaná stock population and had spinal curvature. These individuals were crossed into the pedigree and their offspring have been recorded as a sub lineage within *curveback*. Pedigree was generated using Pedigree Viewer (http://www-personal.une.edu.au/~bkinghor/pedigree.htm).
**Figure 1.3: The *curveback* phenotype**

**A:** The original curve phenotype occurred exclusively on the sagittal plane (red arrows) of the fish (this curve magnitude would be scored as the highest on the qualitative scale). **B:** With selection for the highest curve magnitude, curvature evolved so that there is deviation on the coronal plane of the fish (red arrows) in addition to the sagittal curve. There is also evident axial rotation so that the spine resembles a helix within the body of the fish. Photos taken with a digital camera on a light table under 3X magnification. Scale shown in mm.
In some of the families for which there was selection for curves of high magnitude, the typical curveback phenotype (sagittal lordosis and kyphosis) changed. Individuals with morphological change were selected and a small number of their progeny showed a similar morphology. A-C show related individuals, and E-H show related individuals. All fish shown are adult females. Photos taken with a digital camera on a light table, scale in mm.
Table 1.1: Summary of human genetic studies after 1990

<table>
<thead>
<tr>
<th>candidate genes</th>
<th>results</th>
<th>year</th>
<th>reference</th>
<th>sample size/composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1 &amp; COL1A2</td>
<td>no association</td>
<td>1992</td>
<td>Carr, et al.</td>
<td>3 pedigrees</td>
</tr>
<tr>
<td>FBN1, elastin, COL1A2</td>
<td>no association</td>
<td>1996</td>
<td>Miller, et al.</td>
<td>11 pedigrees (52/96 with IS)</td>
</tr>
<tr>
<td>aggrecan</td>
<td>no association</td>
<td>2002</td>
<td>Zorkol'tseva, et al.</td>
<td>33 families</td>
</tr>
<tr>
<td>aggrecan</td>
<td>associated</td>
<td>2006</td>
<td>Sharipov, et al.</td>
<td>unknown (report in Russian)</td>
</tr>
<tr>
<td>aggrecan</td>
<td>no association</td>
<td>2006</td>
<td>Marosy, et al.</td>
<td>58 families</td>
</tr>
<tr>
<td>FBN3</td>
<td>no association</td>
<td>2008</td>
<td>Cao, et al.</td>
<td>560 girls (273 with IS)</td>
</tr>
<tr>
<td>pectus excavatum</td>
<td>associated</td>
<td>2009</td>
<td>Gurnett, et al.</td>
<td>1 family</td>
</tr>
<tr>
<td>mantrilin-1 promotor</td>
<td>associated</td>
<td>2009</td>
<td>Chen, et al.</td>
<td>369 (197 with IS)</td>
</tr>
<tr>
<td>VDR, ER, CYP17</td>
<td>no association</td>
<td>2002</td>
<td>Inoue, et al.</td>
<td>304 girls with IS</td>
</tr>
<tr>
<td>ER (Xbal &amp; Pvull sites)</td>
<td>Xba1 associated with severity</td>
<td>2004</td>
<td>Inoue, et al.</td>
<td>304 girls with IS</td>
</tr>
<tr>
<td>IGF-1</td>
<td>associated with severity</td>
<td>2006</td>
<td>Yeung, et al.</td>
<td>733 girls (506 with IS)</td>
</tr>
<tr>
<td>VDR</td>
<td>maybe associated</td>
<td>2007</td>
<td>Xia, et al.</td>
<td>286 girls (164 with IS)</td>
</tr>
<tr>
<td>CAM1, GHR</td>
<td>maybe associated</td>
<td>2007</td>
<td>Zhuang, et al.</td>
<td>60 (24 girls and 6 boys with IS)</td>
</tr>
<tr>
<td>MTNR1B promotor</td>
<td>associated</td>
<td>2007</td>
<td>Qiu, et al.</td>
<td>776 (472 with IS)</td>
</tr>
<tr>
<td>GH promotor</td>
<td>no association</td>
<td>2008</td>
<td>Qiu, et al.</td>
<td>458 (265 with IS)</td>
</tr>
<tr>
<td>MTNR1A</td>
<td>no association</td>
<td>2008</td>
<td>Qiu, et al.</td>
<td>453 girls (226 with IS)</td>
</tr>
<tr>
<td>TPH1, AANAT</td>
<td>TPH1 associated</td>
<td>2008</td>
<td>Wang, et al.</td>
<td>211 (103 with IS)</td>
</tr>
<tr>
<td>CAM1, ESR1</td>
<td>maybe associated</td>
<td>2008</td>
<td>Zhao, et al.</td>
<td>167 (67 with IS)</td>
</tr>
<tr>
<td>Xbal</td>
<td>maybe associated</td>
<td>2009</td>
<td>Zhang, et al.</td>
<td>358 (218 with IS)</td>
</tr>
<tr>
<td>ERalpha</td>
<td>associated</td>
<td>2009</td>
<td>Esposito, et al.</td>
<td>278 girls (104 with IS)</td>
</tr>
<tr>
<td>SNTG1</td>
<td>associated</td>
<td>2004</td>
<td>Bashiarde, et al.</td>
<td>1 family</td>
</tr>
<tr>
<td>CHD2</td>
<td>associated</td>
<td>2008</td>
<td>Kulkarni et al.</td>
<td>1 female, Chd2(+/-m) mice</td>
</tr>
<tr>
<td>DPP9</td>
<td>no association</td>
<td>2008</td>
<td>Qiu, et al.</td>
<td>807 girls (571 with IS)</td>
</tr>
</tbody>
</table>

Chromosomal Region

<table>
<thead>
<tr>
<th>chromosomal region</th>
<th>status</th>
<th>year</th>
<th>reference</th>
<th>note</th>
</tr>
</thead>
<tbody>
<tr>
<td>8q: pericentric inversion</td>
<td>N/A</td>
<td>2002</td>
<td>Bashiarde, et al.</td>
<td>1 family (3 generations)</td>
</tr>
<tr>
<td>17p (20cM interval)</td>
<td>N/A</td>
<td>2002</td>
<td>Salehi, et al.</td>
<td>1 family (3 generations)</td>
</tr>
<tr>
<td>19p</td>
<td>N/A</td>
<td>2002</td>
<td>Chan, et al.</td>
<td>7 families</td>
</tr>
<tr>
<td>Xq</td>
<td>N/A</td>
<td>2003</td>
<td>Justice, et al.</td>
<td>202 families (1198 individuals)</td>
</tr>
<tr>
<td>CH6,9,16, and 17</td>
<td>N/A</td>
<td>2005</td>
<td>Miller, et al.</td>
<td>202 families (1198 individuals)</td>
</tr>
<tr>
<td>19p</td>
<td>N/A</td>
<td>2006</td>
<td>Alden, et al.</td>
<td>202 families (1198 individuals)</td>
</tr>
<tr>
<td>5p, 13q</td>
<td>N/A</td>
<td>2006</td>
<td>Miller, et al.*</td>
<td>7 families (53 individuals)</td>
</tr>
<tr>
<td>9q and 17q</td>
<td>N/A</td>
<td>2007</td>
<td>Ocaka, et al.</td>
<td>25 families</td>
</tr>
<tr>
<td>12p</td>
<td>N/A</td>
<td>2009</td>
<td>Raggio</td>
<td>7 families</td>
</tr>
</tbody>
</table>

*linked regions coincide with kyphoscoliosis in a subset of families from a larger IS cohort.
CHAPTER 2: TELEOSTS AS MODELS FOR HUMAN VERTEBRAL STABILITY AND DEFORMITY*

2.1 Abstract

Vertebral development is a dynamic and complicated process, and defects can be caused by a variety of influences. Spinal curvature with no known cause (idiopathic scoliosis) affects 2-3% of the human population. In order to understand the aetiology and pathogenesis of complex human skeletal defects such as idiopathic scoliosis, multiple models must be used to study all of the factors affecting vertebral stability and deformity. Although fish and humans have many of the same types of offenses to vertebral integrity, they have been overlooked as a resource for study. The most common morphological deformity reported for fish are those that occur during the development of the spinal system, and as with humans, curvature is a common morphological consequence. Here we review spinal curvature in teleosts and suggest that they are an unexploited resource for understanding the basic elements of vertebral stability, deformity, development and genetics. Fish can be a value to vertebral research because they are tractable, have a diversity of non-induced vertebral deformities, and substantial genomic resources. Current animal models lack non-induced deformities and the experimental tractability necessary for genetic studies. The fact that fish are free of an appendicular skeleton should allow for analysis of basic spinal integrity without the biomechanical constraints observed in quadrupedal and bipedal models. To illustrate the point we review human idiopathic scoliosis and the potential contribution teleosts can make for the identification of causes, risk factors, and treatment options.
2.2 Introduction

Many factors can compromise the integrity of the vertebral system. Because it is integrated into the body both structurally and functionally, defects of the spinal system have the potential to produce complex phenotypes for which the primary causes are convoluted by secondary phenotypes. Models used so far are insufficient to study all of the factors affecting spinal stability and deformity, and thus many of the factors that maintain basic vertebral integrity are unstudied. With regard to complex disorders in humans, multiple models are critical for the investigation and manipulation of aetiological factors. The value of teleosts as tools for biomedical research is well recognized. In this review we discuss the potential of teleost fish as a tool to fill-in some of the gaps in human vertebral research. Defects of the vertebral system in fish and humans have many of the same causes including genetic, physiological (e.g. calcium regulation), developmental (e.g. fused vertebrae) and infectious (viruses, parasites) (Table 2.1). In fact, the most common type of deformity seen in fish is vertebral, and most of these occur during development. Fish systems could be of enormous benefit to vertebral research because they are tractable, exhibit a diverse range of deformities, are free from an appendicular skeleton, and substantial genomic resources have been developed for several species. Here we review spinal curvature in model teleosts and suggest that they are an unexploited resource for understanding the basic elements of vertebral stability, deformity, development and genetics. We illustrate this point by our research into
the mutant guppy *curveback* (Figure 2.1) as a model for human familial/idiopathic curvature.

### 2.2 Spinal curvature in humans

The human spine is normally straight in the coronal plane. At birth there is a slight kyphosis (dorsally directed sagittal curve) from the crown of the head to the buttocks. As a consequence of bipedalism four natural curves develop in the sagittal plane. Control of the head induces cervical lordosis (ventrally directed sagittal curve), and standing causes a lumbar lordosis. Therefore in the normal spine there are cervical and lumbar lordoses and thoracic and sacral kyphoses (Dickson, 2004). There is natural variation among individuals for the magnitude of sagittal curves. Exaggerations of normal curvature are considered abnormalities when they become dysfunctional.

In addition to exaggerations of innate curvature, aberrant spinal curvature can be caused by a variety of influences. Consequently, deformities are classified according to their presumed aetiology (Terminology Committee of the Scoliosis Research Society: a glossary of scoliosis terms. 1976). Each aetiological category is defined by characteristics that are imposed by the pathophysiology of an underlying condition. Structural curves are those that have the ability to progress during growth (Dickson, 2004). Congenital anomalies (curvature caused by vertebral malformation), idiopathic curvature (curvature with no apparent cause), neuromuscular disorders, neurofibromatosis, connective tissue disorders, and skeletal dysplasia are structural curves specific to the paediatric age group. Although not recognized as an aetiologic category, (the
Scoliosis Research Society) genetics have been identified as the underlying aetiology in an increasing number of structural curves. Such is the case for Marfan syndrome, a disorder affecting the connective tissue (Kumar and Guille, 2001; Coucke et al., 2006), and Friedreich's ataxia, a disorder affecting neurological control (Labelle, 2001; Pandolfo, 2006). For curvature such as idiopathic scoliosis and Scheuermann's kyphosis, a genetic basis is widely accepted but there is still no established aetiology (Ogilvie, et al., 2006; Damborg, et al., 2006).

2.3 Idiopathic scoliosis

Human familial/idiopathic scoliosis represents the largest subgroup of human spinal curvatures. Eighty percent of all structural curves are idiopathic (IS [MIM 181800]) making IS the most common form of spinal deformity in humans. Generally, its incidence among otherwise healthy school-age children is between 0.15%-10.0% (Lonstein, 1995; Axenovich, et al., 1999; Reamy, et al., 2001; Asher and Burton, 2006). Since the deformity was first described by Hippocrates, the diagnosis, cause and treatment of IS have been the focus of a great deal of research. However, phenotypic variability, curve pathogenesis that coincides with dynamic growth, and the lack of an animal model with non-induced curvature has made it difficult to identify the aetiology. The diagnosis is further complicated by the fact that (without consideration of teleosts) idiopathic-type curvature appears to be exclusive to humans. There is a strong suggestion that IS is not a homogeneous group, but one that is composed of several other subgroups, the exact aetiologies of which have not been described.
Generally, it is characterized structural curvature with no vertebral defects that typically occupies all three planes of the body (the majority of curves exhibiting coronal deviation). The deformity has unique characteristics such as, phenotypic variability for age of curve onset, curve morphology, and rate of curve progression, and a gender bias for girls to develop severe curves. Because of variable ages for curve onset, the deformity is classified on the basis of the child's age when curvature is first identified; for infantile idiopathic scoliosis (IIS) onset is before the age of four years, juvenile idiopathic scoliosis (JIS) from age 4 to 9 years, and adolescent idiopathic scoliosis (AIS) occurs between 10 years and skeletal maturity (James, 1954). Generally, the majority of curves that appear during infancy resolve before sexual maturity is reached, while juvenile onset IS tends to be progressive. Adolescent onset is the most prevalent type of IS. The fact that most cases of IS are diagnosed between 10-14 years may reflect that AIS is present in a latent form in younger children who manifest a minor degree of curvature that develops slowly (Stirling, et al., 1996).

Hypotheses for the cause of idiopathic curvature include differences in growth patterns, connective tissue abnormalities, asymmetries in the central nervous system, distribution of melatonin and calmodulin, hormonal variation, ectomorphy/spinal slenderness, diet and posture. Familial clustering (Garland, 1934; Wynne-Davis, 1968; Riseborough and Wynne-Davis, 1973) and concordance among monozygotic twins (Kesling and Reinker 1997; Andersen, et al., 2004) has established a genetic basis as a primary causative factor (Beals, 1973; Harrington, 1977), although the mode of inheritance is still a matter of
debate. One important outcome of understanding the genetics underlying idiopathic curvature would be earlier detection. There is not an established method for detection of curves in growing children. A recent review of screening methodology showed that the most common method of curve detection was by family or friends. The majority of cases detected by such means presented a curve of such magnitude spinal fusion surgery is necessary (Fazal and Edgar, 2006). Early detection of curvature may mitigate curve progression so that non-operative therapies are more effective.

The deformity is complicated by biomechanical and developmental and possibly genetic variability between individuals (Miller, et. al., 2006; Ogilvie et al., 2006). Phenotypic components such as curve magnitude and morphology, progression risk, and time of onset can vary within a pedigree. Segregation analysis using human pedigrees has suggested a single gene for the major determinant of IS (Axenovich, et al., 1999). However, the study only uses pedigrees with a proband having a reported curve magnitude of at least 15° (Cobb angle; unit of measure for lateral deviation of spine in human IS). Therefore only a portion of the phenotype has been considered in pedigree studies, which is likely to be an effect of detection difficulty for curves of slight magnitude. There is little concordance for identified loci among linkage studies using human pedigrees (Carr, et al., 1992; Miller, et al., 1996; Bashiardes, et al., 2002; Chan, et al., 2002; Inoue, et al., 2002; Salehi, et al., 2002; Justice, et al., 2003; Alden, et al., 2006; Ogilvie et al., 2006; Marosy, et al., 2006; Miller et al., 2006; Sharipov et al., 2006; Wu et al., 2006). To date, the genetics underlying
idiopathic curvature have not been identified. This is most likely a consequence of several factors, including: inconsistent pedigree construction between human studies, an arbitrary consensus threshold for proband curve magnitude that may obscure true heritability, and the lack of a genetic model.

It is hypothesized that the three-dimensional nature of IS is a consequence of secondary biomechanical modifications to a primary lordosis in the mid-sagittal plane (Roaf, 1966; Perdriolle and Vidal, 1987; Millner and Dickson, 1996; Ganey and Ogden 2001; Burwell, 2003). The human spine must bear the load of the head while tethered at the pelvis, and so theories such as 'column buckling' or the Hueter-Volkmann principle have been used to explain the three-dimensional nature of human curvature (Millner and Dickson, 1996; Schultz et al., 1984; Burwell, 2003; Stokes, et al., 2006). If primary factors cause an imbalance on the sagittal plane of the vertebral column, then it is predisposed to collapse onto the coronal plane and in some cases, vertebral rotation is encouraged by the ribs. According to Millner and Dickson (1996), although idiopathic scoliosis is a three-dimensional deformity, the problem is more one of front-back asymmetry as opposed-to right-left. This idea has been supported by an observed loss of coupling in the longitudinal growth between the anterior column and the posterior column (Cheng, 2003; Guo, 2003).

In order to understand components of the IS deformity phenotype, the spine biomechanics has been extensively modelled in vitro by computer and three-dimensional reconstruction using mammals such as rabbits, goats, and pigs. However, because curve pathogenesis coincides with development,
simulation modelling can elucidate only a fraction of the entire deformity. Rates of curve progression and biomechanics are mitigated by individual morphology, relative growth rate, and age of curve initiation (Stokes and Windisch, 2006). Heterogeneity among patients in different studies has further complicated an innately variable morphology (Escalada et al., 2005). Attempts to classify a curve’s morphology before skeletal maturity can be complicated by progression of the primary curvature and/or the development of secondary curves (Robinson and McMaster, 1996).

2.4 Animal models for human idiopathic scoliosis

The suggestion that human biomechanics or physiology can modify primary aetiological factors, and the possibility that human IS may consist of many aetiological subgroups, implies that more than one type of model will be necessary to address all levels of the complex IS phenotype. Models used to study the pathogenesis of IS curvature rely on induced curvature and are therefore limited (Braun, et al., 2006); it is controversial whether conclusions made from such experiments relate to primary or secondary influences for curvature (reviewed in Kawakami et al., 1999). In animals with non-induced curvature the type of scoliosis studied is congenital to other conditions (e.g. hens, Ruble, et al., 2002; scoliosis mouse, Adham, et al., 2005), or occurs in sexually mature animals (Riggins, et al., 1977; Naique, et al., 2003), and thus doesn’t reflect the human condition. Because heritable, developmental, idiopathic spinal curvature has been exclusive to humans, it has been hypothesized to be a consequence of bipedalism (Burwell et al., 1992; Machida, et al., 1999).
Current developmental modelling is further complicated by the fact that most of the animals used thus far are quadrupedal. In these animals, the structure of the spine and the centre of gravity are radically different from humans (Mack and Greenberg, 1990; Kawakami et al., 1999; O'Kelly, et al., 1999). Despite the fact that the vertebral anatomy of the bird is grossly different from that of non-avian vertebrates (e.g., no intervertebral discs, multiple fused vertebrae), the pinealectomized chicken is the most common animal model of IS. This is largely because pinealectomy induces spinal curvature in most chickens, and because chickens are bipedal. Pinealectomy in hamsters and monkeys does not induce curvature (O'Kelly et al., 1999; Cheung, et al., 2005). Pinealectomized rats do not demonstrate spinal curvature unless they are made bipedal by removing the tail and two front legs (Machida et al., 1999). Likewise, mice made melatonin-deficient without pinealectomy do not show spinal curvature unless they are made bipedal (Machida et al., 2005). Due to conflicting results between human and chicken studies, whether melatonin plays a role in the formation of curvature is also a topic of debate (Bagnall et al, 1996). The forces that act on a bipedal spine are different than those acting on a quadruped. It is not clear whether genes for idiopathic-type curvature are present in terrestrial animals but constrained by quadrupedal biomechanics, as the studies using the bipedal rat suggests, or if the primary aetiology is exclusive to humans. As all animals used in orthopaedic modelling thus far are quadrupedal, the latter assumption has been favoured.
Fish are ideal models for studying vertebral deformities. The most common deformities among teleosts are those that occur during development of the spinal system, and humans and fish share many aetiological factors for non-idiopathic types of curvature (Table 2.1), demonstrating general homology for spinal deformity. Importantly, pinealectomy in the guppy and salmon induces spinal curvature with a physiological response the same as in chickens (Plugfelder, 1953; Mayer, 2000; Fjelldal, et al., 2004), the most common model of IS. Furthermore, the teleost spine is not weight-bearing, due to buoyancy provided by the swim bladder combined with the density of water (Fjelldal, et al., 2004). Therefore, fish present the opportunity to investigate factors that influence curvature without the constraints of quadrupedal or bipedal biomechanics. Factors that influence human curvature such as gravity, vertical loading, rib association and lumbar tethering can be omitted from the equation of possible influences for curve progression, thereby illuminating important primary correlates.

2.5 Spinal curvature in model teleosts

The abundance of spinal deformities among fish suggests their innate power to reveal important variables for basic spinal stability and deformity. To illustrate the point, in figure 2 we review 63 studies from over 20 species showing vertebral deformities. Types of deformity commonly seen are lordosis, kyphosis, scoliosis, and broken/cracked vertebrae. Causes can be physical injury, environmental, nutritional, infection, or genetic (Figure 2.2). Until recently, the ontogeny of fish spinal curvatures in non-model teleost species has received
relatively little attention and most studies have been largely descriptive. Consequently, few deformities have been explored beyond the level of association with particular causative factors (Brown and Nunez, 1998).

Among Euteleosts, the model fish species guppy (*Poecilia reticulata*), medaka (*Oryzias latipes*) and swordtail (*Xiphophorus maculatus*) have been shown to exhibit similar (although not characterized in swordtail) spontaneous spinal curvature mutants (Figure 2.3). Curvature in these closely related teleosts is similarly described as a primary dorso-ventral deviation (lordosis and/or kyphosis), with some fish exhibiting coronal curvature and vertebral rotation. Clear and stain of medaka wavy and guppy curveback mutants shows normal vertebrae, and as with human idiopathic curves, the vertebral centra are often warped at the point of greatest curvature (curve apex) (Takeuchi, 1960; Gorman and Breden, unpublished data). Computed tomography scans of curveback guppies show no vertebral fusion or breaking (Gorman et al., in press). The fused medaka mutant and the stubby and palla guppy mutants are similarly described as fusion of a variable number of vertebrae that causes shortening of the spine (Aida, 1930; Schultz, 1963; Lodi, 1978). Historical characterization of non-induced spinal curvature in the guppy and medaka has been largely descriptive, with particular focus on inheritance, calcium and phosphorous ratios in the vertebrae, and tissue analysis (Harrison, 1941; Rosenthal and Rosenthal, 1950; Rosenthal, 1953; 1954; 1958; Takeuchi, 1960; Marquet and Sobel, 1969).

A review of the curve phenotypes in figure 2.3 suggests that multiple genes are associated with curvature, and that some of the genes may be
conserved between medaka and guppy. The wavy medaka, hunchback swordtail, and curveback guppy phenotypes have a very similar curve morphology (primary lordosis/kyphosis), inheritance pattern, as well as trait expressivity (variable penetrance for magnitude of lordosis and kyphosis). Conservation of gene function has been confirmed among orders of Euteleosti (Sato et al., 2000), and so it is not unreasonable to assume that the guppy, swordtail, and medaka share genes involved in the spinal curvature phenotype. Both the guppy and medaka genomes have independently been shown to have strong homology with the swordtail genome (Brummell, et al., 2006; Ju, et al., 2006). The fact that guppy and medaka also exhibit the heritable fused/ stubby vertebral mutants with strong phenotypic and genetic similarity, further suggests conserved genes for vertebral development and function among these species. Interestingly, the zebrafish model is more distantly related than the guppy and medaka (i.e., is not a Euteleost: Crollius and Weissenbach, 2005), and has no incidence of heritable, idiopathic-type curvature. This may be a reflection of genetic differences, as zebrafish have different copy-numbers and expression patterns for important developmental genes than medaka and pufferfish (Schartl, 2004; Gajewski, et al., 2006; Kurosawa, et al., 2006).

Differences in curve morphologies and inheritance patterns between species and/or strains of fish could either indicate modifying genes or modified expression patterns of the same genes involved in the phenotype. With all curve phenotypes, genetic complexity is apparent in curve morphology and the time of curve onset. As seen in figure 2.3, there are single factor recessive and complex
inheritance patterns observed in different populations of swordtail and guppy. Analysis by Harrison (1941) of *hunchback* guppies revealed two curve phenotypes with different inheritance patterns, although they segregated in the same pedigree. The curve phenotype associated with complex inheritance demonstrated a greater propensity for coronal deviation, and later onset (around 50 days past birth), than curves that were apparent at birth. Curves present at birth showed Mendelian recessive inheritance and curvature that increased steadily with growth. Studies by Rosenthal (1951) and Goodrich (1943) describe Mendelian recessive inheritance for curvature present at birth in guppies, but mention individuals for whom curvature matures after birth and exhibits a different and more complex inheritance pattern. However, no further analysis was published. Ando et al (1995) demonstrated that different curve morphologies within strains of guppies are correlated to differential segregation of genetic markers, demonstrating that multiple genes account for some curve phenotypes. Furthermore, there are two deformities involving fused vertebrae in the guppy, one with dominant inheritance, and one recessive. These studies demonstrate the genetic complexity of basic spinal stability, reinforcing the contribution of teleosts to the study of the genetic basis of spinal deformities.

The studies indicated in figure 2.3 indicate that spinal curvature does not hinder fitness in adults (e.g., lifespan, feeding, activity). Although not correlated to the propensity for curvature, the eggs of the 'wavy' medaka mutant strain are "weaker" than normal fish eggs and tend to die during embryonic development (Takeuchi, 1960). There are no differences in the embryonic
development of the lordotic and normal guppies (Rosenthal, 1953). Rosenthal’s report (1951) of semi-lethalism in the male lordotic guppy is questionable, because he compares the inbred lordotic guppy strain to wild-caught guppies. Our experience in the lab with guppies is that fecundity decreases in general as laboratory strains become more inbred, and thus the lower fecundity may reflect the inbreeding during the maintenance of the lordotic lines.

Chemical analysis in curved fish has focused on calcium and/or phosphorus levels in vertebral structures, and embryonic or larval calcification (Harrison, 1941; Rosenthal, 1950, 1953, 1954, 1958; Rosenthal et al., 1958; Takeuchi, 1960). Although calcium and phosphorus levels during guppy embryogenesis are not different among lordotic guppies compared to normal (Rosenthal, 1953), Rosenthal suggests that there is an alteration of calcium metabolism in the vertebrae but not muscle of adult curved fish (1954, 1958). Abnormal calcium metabolism has been suggested as a possible component of human IS (Cheung, 2006). Excess crystalline inclusions were found in the endoplasmic reticulum and nuclear envelop of the spinal cord oligodendroglia of curved guppies (Marquet and Sobel, 1969). This is interesting because virus-like particles have been noted in the paraspinal muscle of humans with idiopathic scoliosis (Webb and Gillespie, 1976; Green et al., 1979). It is possible that the crystalline inclusions in guppies are an indication of excess cellular protein. Whether the human particles indicate a similar cellular process or are an indication that general cellular disruption can cause curvature has not been investigated. Morphological analysis in the wavy medaka such as spine ratios or
vertebral structure was measured. In the 'wavy' mutants the length of the vertebral column is the same as in normal medaka, but vertebral spines are shorter on the convex side of curvature (Takeuchi, 1960).

Growing research in fish vertebral ontogeny, a history of genetic and molecular research regarding spinal curvature of model teleosts, and the growing field of comparative genomics can make model teleosts valuable for human vertebral research. Without many of the complications that have been observed in previous models, fish offer an opportunity to explore the basic elements of vertebral stability and deformity. Comparisons between the human genome and that of non-mammal vertebrates such as fish has proven to be a powerful tool for identifying DNA sequences that have significant functional activity in all vertebrates (Boffelli *et al*., 2004). The fact that humans and fish share many genes with similar tissue and temporal expression characteristics is well established (Aparicio, *et al*., 2002).

**2.6 curveback as a model for human familial/idiopathic scoliosis deformity**

An experimental model that has a strong genetic component and does not rely on induced curvature will have significant value for scoliosis research. Discovery of genes for idiopathic-type spinal curvature and testing the effects of suspected risk factors for curve progression in the guppy might suggest new therapeutic interventions for humans. In IS, the contribution of factors such as biomechanical force, load, growth rate trajectories, hormone levels, spinal length, vertebral distortion, and sex has been debated. Using the tractable guppy model,
we will be able to test each factor as a separate hypothesis and determine whether it contributes to the progression of curvature. We have shown how developmental trends in the curveback lineage parallel the human condition of familial/idiopathic scoliosis (Gorman, et al., 2006). Our results show the guppy's potential as a tractable model organism for investigation into genetic and developmental factors that influence spinal curvature.

Spinal curvature in the *curveback* guppy develops after birth, when the skeleton is fully ossified. Guppies are livebearers, and are born about 3 weeks post fertilization. The primary force/load on the guppy spine is directed in a cranial to caudal (anterior to posterior) direction, principally due to motion through water, so that the orientation of primary curvature is perpendicular to the load (similar to human IS). Very importantly, CT scans and clear and stain analysis of skeletons shows that curvature is not caused by vertebral malformation, making *curveback* the first animal model for human IS with non-induced curvature. As in humans, there is a female bias for extreme/progressed curves, despite the fact that there is no apparent gender bias at the time of curve onset. In both the human and *curveback* guppy populations the majority of curves are slight in magnitude, while some progress to a great extent, and there is a class of curves that resolve before the individual reaches skeletal maturity. Variability in the *curveback* guppy population for age of curve onset, propensity to demonstrate progression of curvature, and curve magnitude resembles that described for the human population (Gorman, et al., 2006). The observed distribution for curve magnitude is continuous in both the *curveback* and human IS populations (Miller,
The pattern of curve progression for guppies is similar to humans in that the angle grows in spurts instead of a linear progression through development (Escalada, et al., 2005). These similarities between the guppy curveback mutant and human IS are summarized in Table 2.2.

The guppy has been an important laboratory organism for genetic analysis since the 1920’s (Winge, 1922). A growing number of genomic resources make gene identification possible in the near future. Candidate genes identified in teleosts can be screened in human pedigrees for association with IS. Much of the work demonstrating human/teleost homology has been done with medaka, often in systems implicated in the IS phenotype (e.g.: pineal gland: Alunni, et al., 2004; neuronal development: Okubo, et al., 2006; somitogenesis: Gajewski, et al., 2006; bone formation: Wagner, et al., 2003; Renn, et al., 2006; vertebral column formation: Ohtsuka, et al., 2004; Yasutake, et al., 2004). Medaka is closely related to the guppy, and several lines of evidence (such as same mode of inheritance, and similar trait morphology and expressivity) suggest that the mutant wavy is genetically similar to curveback in the guppy. Using two models with similar curve phenotypes will increase the likelihood of identifying genes conserved in human developmental processes that are involved in IS.

2.7 Conclusion

The growing number of sequenced genomes and overall genomic resources available for fish has made them an important tool for the investigation of human genetics, development and disease (e.g. reviews from: Danzmann and Gharbi, 2001; Prince and Pickett, 2002; Wittbrodt, et al., 2002; Boffelli, et al.,
Experimental studies of spinal deformities in fish are an unexploited resource capable of answering questions of vertebral development, basic stability, and deformity. The spinal column of fish is free from an appendicular skeleton, and is not weight-bearing, allowing for an opportunity to investigate the factors that maintain the vertebral skeleton without the constraints of quadrupedal or bipedal biomechanics. Complicating factors such as gravity and load can be omitted from the equation of possible influences for curve progression, thereby illuminating important factors for basic spinal stability.

By using model teleosts for understanding conserved features of vertebral stability, research into vertebral deformities will be able to distinguish between primary and secondary aetiological factors of deformity phenotypes, something that has been very difficult with the accepted animal models so far. We expect that with model teleosts it will be possible to manipulate suspected aetiological correlates such as hormone and mineral (e.g. calcium) levels. With inbreeding not only can inheritance be investigated in detail, but also selective breeding will allow for the creation of families enriched for genes contributing to deformities. Mapping crosses, experimental crosses, and transgenics are all possible in model teleosts, making them a valuable tool for the understanding of the development of the vertebral system and the aetiology of deformities.

2.8 Acknowledgments

We acknowledge the advice and encouragement of S. Tredwell of the Department of Orthopaedics, British Columbia Children's Hospital, and thank Julian Humphries and Tim Rowe at the University of Texas High-Resolution X-ray
Computed Tomography Facility, and support from Natural Sciences and

2.9 Literature Cited


Harrison, R.W., 1941. A contribution to the genetics of *Lebistes Reticulatus* the nature and inheritance of ‘Hunchback’. Thesis, Wesleyan University, Connecticut, USA.


Stemshorn, K.C., Nolte, A.W. and Tautz, D., 2005. A genetic map of Cottus gobio, Pisces, Teleostei. based on microsatellites can be linked to the physical map of Tetraodon nigroviridis. Journal of Evolutionary Biology 18, 1619-1624.


Winge, O., 1927. The location of eighteen genes in *Lebistes reticulatus*. J. Genetics 18, 1-43.


Figure 2.1: The *curveback* phenotype

**A:** The *curveback* phenotype is a primary anterior lordosis (L) and a secondary posterior kyphosis (K) occurring on the sagittal plane. CT scan shows no vertebral breaks or fusion associated with curvature (some individuals demonstrate coronal deviation—not shown). **B:** Normal fish with sagittal plane and dorsal/ventral axes shown. Digital photos of anaesthetized adult females taken on a standard light table under 3X magnification, CT scans consist of 350 0.4mm slices. Inset images taken at the University of Texas High-Resolution X-ray CT Facility (datasets of scans available for view at http://www.digimorph.org/index.phtml).
Figure 2.2: Causes of Spinal Deformity in Fish

Spinal deformities in fish can be caused by a variety of influences. Shown here are general categories for insults to vertebral integrity. Because spinal deformities are so prevalent among fishes, this is not an exhaustive list of publications.

Environmental

- **Electric current**: trout- DeVore and Eaton, 1983.

Nutrition

- **Amino acid deficiency**: salmon- Post, 1933; Akiyama, et al., 1966.

Physical Injury

- **Pinealectomy**: guppy- Plugfelder, 1953; salmon- Mayer, 2000; Fjeldal, et al., 2004.

Genetic/heritable (laboratory & hatchery strains)

Figure 2.3: A summary of the research conducted on model teleosts with regard to heritable spinal curvature.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutant</th>
<th>Inheritance</th>
<th>Analysis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medaka</td>
<td>fused</td>
<td>recessive</td>
<td>descriptive</td>
<td>Aida, 1930; Ogawa, 1965</td>
</tr>
<tr>
<td></td>
<td>wavy</td>
<td>recessive + modifiers</td>
<td>larval spine dev., spine ratio, eggs</td>
<td>Aida, 1930; Takeuchi, 1960</td>
</tr>
<tr>
<td>Swordtail</td>
<td>hunchback</td>
<td>complex recessive/single factor</td>
<td>descriptive</td>
<td>Gordon, 1934</td>
</tr>
<tr>
<td></td>
<td>(no name)</td>
<td>recessive/ single factor</td>
<td>spine [Ca/P], males sterile</td>
<td>Rosenthal et al., 1958</td>
</tr>
<tr>
<td>Guppy</td>
<td>palla</td>
<td>dominant</td>
<td>inheritance</td>
<td>Lodi, 1978</td>
</tr>
<tr>
<td></td>
<td>stubby</td>
<td>recessive</td>
<td>descriptive</td>
<td>Schultz, 1963;</td>
</tr>
<tr>
<td></td>
<td>hunchback</td>
<td>both recessive and complex observed</td>
<td>descriptive, inheritance</td>
<td>Harrison, 1941</td>
</tr>
<tr>
<td>Lordosis</td>
<td>recessive/ sex linked (females only)</td>
<td>cellular/histological</td>
<td>Marquet and Sobel, 1969</td>
<td></td>
</tr>
<tr>
<td>Curveback</td>
<td>multifactorial</td>
<td>descriptive, development, inheritance</td>
<td>Gorman and Breden</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>not indicated</td>
<td>selection</td>
<td>Ando, et al., 1995</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1: Types of progressive/structural spinal deformities in humans* and reported occurrence in teleosts

<table>
<thead>
<tr>
<th>Deformity types</th>
<th>Observed in fish?</th>
<th>Circumstances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic</td>
<td>yes</td>
<td>Curvature in aquarium guppy.</td>
</tr>
<tr>
<td>Congenital</td>
<td>yes</td>
<td>Many types: seen in cultured and wild fish.</td>
</tr>
<tr>
<td>Neuromuscular</td>
<td>yes</td>
<td>Laboratory zebrafish</td>
</tr>
<tr>
<td>In association with Neurofibromatosis</td>
<td>no</td>
<td>Neurofibromatosis of the Damselfish is a model for NF1 in humans, although there are no reports of spinal curvature.</td>
</tr>
<tr>
<td>Traumatic</td>
<td>yes</td>
<td>In cultured and wild fish.</td>
</tr>
<tr>
<td>Due to infection</td>
<td>yes</td>
<td>*Mycobacterium (M. marinum, M. chelonei, M. fortuitum)</td>
</tr>
<tr>
<td>Due to tumours</td>
<td>yes</td>
<td>In cultured and wild fish.</td>
</tr>
<tr>
<td><strong>Miscellaneous conditions:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>deformities in adults</td>
<td>yes</td>
<td>Curvature in older aquarium fish.</td>
</tr>
<tr>
<td>spondylolistheses</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

*According to the Terminology Committee of the Scoliosis Research Society (1976)
Table 2.2:

<table>
<thead>
<tr>
<th>Parallels between curveback and idiopathic scoliosis phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Born normal, curve develops after birth.</td>
</tr>
<tr>
<td>2) Variability among individuals for rate and propensity of curve progression.</td>
</tr>
<tr>
<td>3) Onset time for curvature is variable.</td>
</tr>
<tr>
<td>4) Curve magnitude increases with age.</td>
</tr>
<tr>
<td>5) Distribution of curve magnitude in population is continuous.</td>
</tr>
<tr>
<td>6) Curvature does not progress past skeletal maturity.</td>
</tr>
<tr>
<td>7) Angle growth is in spurts instead of linear progression.</td>
</tr>
<tr>
<td>8) Curvature does not hinder fitness of individuals.</td>
</tr>
<tr>
<td>9) Female bias for most severe curve.</td>
</tr>
<tr>
<td>10) Incidence of curve that resolves before maturity.</td>
</tr>
<tr>
<td>11) No vertebral fusion or breaking.</td>
</tr>
<tr>
<td>12) Major gene effect.</td>
</tr>
<tr>
<td>13) Complex inheritance</td>
</tr>
</tbody>
</table>
CHAPTER 3: THE MUTANT GUPPY SYNDROME
CURVEBACK AS A MODEL FOR HUMAN HERITABLE
SPINAL CURVATURE*

*A version of this chapter appears as KF Gorman, SJ Tredwell, F Breden 2007.
Spine 32(7): 735-741.
3.1 Abstract

**Study Design:** This study investigated the morphology, pathogenesis and inheritance of idiopathic-like spinal curvature in the guppy syndrome, *curveback*.

**Objective:** To determine whether *curveback* could be applied as a model for the primary factors that contribute to heritable spinal curvature in humans, specifically the etiopathogenesis of human familial idiopathic scoliosis (IS).

**Summary of Background Data:** Although a genetic basis is accepted, phenotypic complexity and the lack of an animal model with non-induced curvature has made identification of IS aetiology difficult. It is well established that humans and fish share many genes with similar tissue and temporal expression characteristics, and comparisons between human and fish genomes have proven to be valuable for understanding the genetics of diseases affecting humans.

**Methods:** The *curveback* lineage of guppies was constructed from a single curved male crossed to a normal female. Offspring (103) from the original cross were scored from birth until death for the presence and magnitude of spinal curvature. Genetic architecture was investigated through selective inbreeding, analysis of the distribution of curve magnitude in the mature population, and assessment of curve dynamics during development. Vertebral detail was assessed by computed tomography.

**Results:** Computed tomography reveals that vertebral breakage or fusion is not associated with the *curveback* syndrome. Inbreeding demonstrates a strong genetic influence on *curveback*, and the distribution of curve magnitude
among adult fish suggests polygenic inheritance. There is a female bias for curves of high magnitude, and for curves that resolve before maturity. There is developmental variability for the age of curve onset, curve progression, and final curve magnitude.

**Conclusions:** Observed parallels between the curveback syndrome and human IS suggests that the guppy model is an unexploited resource for the identification of primary aetiological factors involved in curvature. As models for biomedical research, teleosts offer great potential regarding spinal stability and deformity.

### 3.2 Introduction

Although the diagnosis, cause and treatment of human familial idiopathic scoliosis (IS) has been the focus of a great deal of research, little progress has been made in identifying the aetiology of IS. This is largely due to the extreme phenotypic variability of IS and the lack of an appropriate developmental and genetic animal model. Multiple hypotheses such as differences in growth patterns, connective tissue abnormalities, asymmetries in the central nervous system, distribution of melatonin and calmodulin, hormonal variation, spinal slenderness, diet and posture have all been suggested as causes for IS.

Although it is well accepted that a genetic basis exists for IS, the proportion of phenotypic variation due to genetics and the mode of inheritance of these underlying factors are still a matter of debate.\(^1\)\(^-\)\(^10\) Without a tractable model organism for research, navigation through phenotypic complexity to primary causative factors will continue to be difficult. The authors present data on the
morphology, development, and inheritance of the guppy mutant *curveback*, demonstrating its potential as a tool for understanding genetic and environmental causes of IS.

### 3.3 Materials and Methods

The guppy, *Poecilia reticulata*, is a small, livebearing fish, native to the streams of northeast South America, and is a popular aquarium fish. The guppy has been a model organism for ecology, evolution and genetic research since the 1920's, and was used in this study to investigate the morphology and pathogenesis of the mutant syndrome *curveback*.

**Scoring fish**

The guppy spine is visible without magnification. All fish were scored from the side and above while in a glass view tank 4” long x 2” wide x 3” high. The *curveback* phenotype manifests as a primary sagittal lordosis of variable magnitude with some individuals exhibiting posterior kyphosis and/or coronal deviation (Figure 3.1). Since all affected individuals exhibit sagittal curvature, the degree of lordosis as a standard measure of curvature was used.

**Qualitative**

The magnitude of lordosis observed in the *curveback* population was categorized into five general classes: type 0 (normal), and curve types 1-4, with 4 being extreme curvature (Figure 3.2). There is also a type of curvature that develops shortly after birth but resolves before maturity; this was scored as a sixth qualitative category (type 5).
Quantitative

Lordosis was measured in adult fish that had been anesthetized with ice water (7 second exposure) and then photographed with a digital camera (Toshiba PDR-3310, NYC, USA) under 3X magnification on a standard light table. Using Image-J (NIH Image)\textsuperscript{11}, a body midline was drawn on digital images between the tip of the caudal peduncle (CP) and the centre of the occipital orbit (OC) (Figure 3.3). A perpendicular line was then drawn from the midline to the apex of the lordotic curve (A). Anterior curve ratio was defined as the ratio of the length of the perpendicular line (magnitude of lordosis) relative to the length of the fish (CP to OC). This measure is similar to that used by Cheung \textit{et al.} (2006) to measure lateral deviation of apical vertebra.\textsuperscript{12} The distribution of the anterior curve ratio in the population was analyzed using the Kolmogorov-Smirnov test.

Skeletal imaging

Although the guppy spine is visible without magnification, individual vertebrae cannot be discerned. Vertebral detail was observed on a representative normal and a type 4 curved fish by CT scans at the University of Texas High-Resolution X-ray Computed Tomography Facility\textsuperscript{13}, as described in Ketcham and Carlson, (2001).\textsuperscript{14} Each three-dimensional scan consists of 350 slices 0.4mm thick, which would allow detection of malformed, fused, or cracked vertebrae.
Establishment and maintenance of curveback lineage

The curveback lineage originated from a curved male crossed to a normal female, followed by full-sib mating. Both founders were from a population collected in Cumaná, Venezuela\textsuperscript{15}, which had been maintained as a lab stock for 4 years. To test for recessive genetic components of the syndrome, 2 sib-mate pairs (A and B) were made from F\textsubscript{1} progeny. To test for recessive components in the mother's genotype, a F\textsubscript{1} male was backcrossed to his mother.

Based on the qualitative scores described in Figure 2, we made the following crosses from the F\textsubscript{2} progeny from sib-mate pair A: i- curve type 3 male to normal female; and ii- type 4 female; iii- type 4 male to type 4 female; iv- type 1 male to normal female. From sib-mate pair B: i- type 2 male to type 4 female; ii- normal male crossed to normal female.

Selection based on curve magnitude was carried to the fifth filial generation in order to create families enriched with genes for observed curve magnitudes, detect possible genetic linkage for different curve magnitudes, reveal recessive alleles, and determine consistency in inheritance of curvature (i.e. amount of variation due to genetic background or modifier genes).

Development of curvature

One hundred and three fish were qualitatively scored every 2-4 days from birth until sexual maturity, and then at least once per month as adults until death. Sexual maturity occurs between eight and ten weeks past birth, as is indicated by development of the male gonopodium and darkening of the female gonoporal region (guppies are livebearers; females give birth to live offspring at about 3-4
weeks post-insemination). Skeletal maturity is estimated as the time when the guppy reaches adult morphological proportions.

3.4 Results

Curveback morphology

Of 25 adult fish with lordosis, 8 also had secondary kyphosis. Six of the 8 were of curve type 3 or 4. Of the 17 adults with only lordosis, 4 had curve types 3 or 4. Visual inspection of 5 (1 male, 4 female) fish (out of 103) exhibiting pronounced lordosis, kyphosis and coronal curvature demonstrate that vertebral rotation can be part of the curveback syndrome.

Computed tomography (CT) scans reveal that vertebral anomalies, breakage, or fusion is not associated with curvature (Figure 3.4). The three-dimensional roll-scans allowed for examination of 18 complete precaudal and caudal vertebrae in a normal and severely curved fish (datasets of scans available for view at http://www.digimorph.org/index.phtml). The scans show that vertebrae involved in curvature are wedge shaped, with the vertebrae at the apex of curvature being most severely warped. Although rotation of the vertebral column is evident in scans of the curved fish, a means to quantify such a character has not yet been optimized.

Curveback Development

Curvature develops after birth and generally does not progress after skeletal maturity. Of the 103 fish scored from birth past sexual maturity, 61% (n=63) demonstrated curvature during development. Of these, 79% (n=50) were
born with normal spines and developed curvature within the first 2 weeks past birth. The remaining 21% of the fish (n=13) were born with curved spines that resolved to normal or nearly normal before maturity. Whether curvature resolves does not depend on the magnitude of curvature during development. There is a female bias for curves of high magnitude (15% of scored females have severe curvature compared to 4% of scored males, N=103), and for resolving curves (18.5% female vs. 8% male, N=103), and a male bias for slight curvature (type 2) (Figure 3.5).

All curves begin small in magnitude, but the initial magnitude as well as the age of onset is variable both within and between families. Fish born on the same day were scored together, which enabled detection of individual differences for onset and progression. The curves of greater amplitude begin within 4 days past birth, while curves that initiate after 7 days tend not to progress past type 2. Progression of curvature is periodic, oscillating between periods of increase or decrease and relative stasis (Figure 3.6). The general pattern for progression of curvature during growth is the same regardless of whether the fish is born normal or with curvature.

**Curveback Inheritance**

Our early crosses indicate that recessive genes contribute to *curveback*, and analysis of the distribution of anterior curve ratios among adult fish is not statistically different from normal (P > 0.1). F₁ offspring from the original cross were scored only as adults but showed no curvature, suggesting that the genes controlling the curveback phenotype is autosomal recessive. The proportion of
affected curveback individuals in the lineage increased in further generations with inbreeding, showing a strong genetic influence on the phenotype. Fifty percent of the F2 offspring demonstrated some degree of curvature during development, regardless of whether they appeared normal as adults. In the F3 generation 58% of the offspring demonstrated curvature during development.

Since the curveback population originated from a single curved male crossed to a non-curved female, and all of the F1 adult progeny (n=7) appeared normal, our assumption was that the male was homozygous recessive for curveback and that the female was homozygous for wild-type genes. We expected that if the mother had any recessive genes for curvature, we would see curved progeny from a cross to her presumably heterozygous son. Three broods totalling 12 offspring had no curved fish, suggesting that genes for curvature are recessive to normal, and that the mother is wild-type. The male was subsequently crossed to a related F2 non-curved female (from F1 sib-sib mate B) and had 12/17 curved progeny, suggesting that the male is heterozygous. Sibmate A yielded 26% curved F2 offspring and sibmate B yielded 12% curved offspring.

Because the F1 and early F2 crosses were conducted before the developmental analysis, animals were scored as adults only for the presence or absence of curvature. We had not detected the subtle ‘type 1’ curve and were unaware of resolving curves until the 7th brood of our F2 generation. When we examine curve scores throughout development and not just at maturity, inheritance appears more complex. From the F2 and successive inbred crosses,
we see that there is no simple pattern of inheritance for curve magnitude, the time
of curve onset and whether curvature resolves or progresses. If curveback were
inherited as a Mendelian recessive trait, then we would expect all curved
offspring from cross ii and iii of sibmate A, which is not the case (Figure 3.7).

3.5 Discussion

With regard to complex developmental disorders in humans, multiple
models are critical for the investigation and manipulation of aetiological factors.
An experimental model with a strong genetic component will have significant
value as a part of scoliosis research. It is well established that humans and fish
share many genes with similar tissue and temporal expression characteristics16-
21, making fish a valuable asset for understanding the genetics of diseases
affecting humans.22-26 Comparisons between human and fish genomes have
proven to be a powerful tool for identifying DNA sequences that have significant
functional activity in all vertebrates.27 Indeed, teleosts show great potential in
general as models for biomedical research regarding spinal stability and
deformity.28-31 Parallels between the curveback syndrome and IS (Table 3.1)
show its potential as a tractable model organism for discovering genetic and
developmental factors that influence heritable spinal curvature in vertebrates.
The guppy has been an important laboratory organism for genetic analysis since
the 1920’s32 with over 400 papers published on the guppy in the last 10 years
alone. A short generation time, the availability of genomic resources, and ease of
care and manipulation make the guppy a tractable organism for vertebrate
research.
**Curveback morphology:** CT scans reveal that the primary lordosis is not associated with vertebral fusion or breakage. Observed distortion of apical vertebrae coincides with typical morphology for human scoliosis where curve vertebrae become warped with progression. Further skeletal analysis using CT and clearing and staining on immature fish will elucidate the timing of structural changes involved in curvature. From such an analysis we can understand whether variables such as vertebral length, width, or morphological asymmetry are common correlates to curvature and/or progression.

Biomechanical differences between human and fish may help to delineate secondary from primary phenotypic components. Pinealectomy in hamsters and monkeys did not induce curvature. Pinealectomized rats do not demonstrate spinal curvature unless they are made bipedal. Consequently, scoliosis is thought to be an effect of bipedalism. What is not clear is whether the primary factors that predispose an animal to curvature are present but constrained by quadrupedal biomechanics, as the study with the rat suggests, or if the primary dysfunction is exclusive to humans, perhaps because the scoliosis-causing mutations are specific to the human lineage. As all animals used in orthopaedic modelling thus far are quadrupedal, the latter assumption has been favoured. Pinealectomy in the guppy and salmon induces spinal curvature with a physiological response the same as in chickens. Furthermore, the teleost spine is not weight bearing due to buoyancy provided by the swim bladder combined with the density of water. Therefore, fish present the opportunity to investigate the factors that influence curvature without the constraints of
quadrupedal or bipedal biomechanics. Factors that influence human curvature such as gravity, load, rib association and lumbar tethering can be omitted from the equation of possible influences for curve progression, thereby illuminating important primary correlates.

For example, it is hypothesized that the three-dimensional nature of human IS is a consequence of secondary biomechanical modifications to a primary lordosis in the mid-sagittal plane.\(^8,39-42\) According to Millner and Dickson (1996), although IS is a three-dimensional deformity, the problem is more one of front-back asymmetry as opposed to right-left. This idea has been supported by an observed loss of coupling in the longitudinal growth between the anterior column and the posterior column\(^43,44\) but still remains a matter of debate. The curveback guppy allows for the investigation of genes that maintain sagittal balance.

**Curveback development:** Shared developmental trends listed in Table 1 demonstrate the possibility of conserved biochemical or physiological processes that may be influencing curve pathogenesis for humans and guppies. Hormones administered to aquarium water can manipulate the rate of sexual maturity and/or growth in order to test for correlation with curvature,\(^45\) and such correlations may help to identify candidate genes.

Incidence of spinal curvature in other animal models is either congenital to other conditions\(^46\) or lacks correlates to human growth or development (i.e. the curvature occurs in the animal after sexual maturity).\(^47,48\) In order to evaluate the pathogenesis of curvature in the context of development, animal experiments in
the past have artificially induced scoliosis. Methods such as tethering, intercostal nerve resection, electrostimulation, irradiation, pinealectomy, magnet implantation, or direct injury to the epiphyseal plate have induced curvature in animals such as the rabbit, chicken, goat, dog, rat, and monkey. However, because the curves are artificially induced, there is controversy over whether conclusions made from these experiments relate to primary or secondary influences for curvature.

**Curveback inheritance:** The mode of inheritance for IS in humans is still a matter of debate. Multifactorial inheritance, autosomal dominant, and X-linked inheritance have all been proposed. Uncertainty is thought to arise in part from a common phenotypic threshold for curvature being set at a 10° Cobb’s angle, which would obscure minimal yet heritable components of the deformity. Human studies that have targeted candidate genes have not successfully correlated a locus or marker with the IS phenotype. Complex inheritance and phenotypic variability resembles that of human familial scoliosis suggesting similarities for genetic architecture. In both human and guppy phenotypes the time of onset, curve magnitude, morphology, and propensity for curve progression vary within a pedigree. In the curveback lineage observed variability for the distribution anterior curve ratios is similar to that described for the magnitude of lateral curvature in human IS, providing support for a multigenic basis. The distribution of qualitative curve types (Figure 3.5) among males and females shows a major gene effect with modifiers, and suggests sex linkage for a portion of the phenotype. The resolving curve type is similar to that described as
human infantile idiopathic scoliosis, but it segregates in the curveback population with all curve types.

The visibility of the guppy spine in vivo makes detection of curve onset and subtleties regarding curve progression easy to score, thereby facilitating more comprehensive pedigree construction. It has been suggested that human adolescent IS is present in a latent form in younger children who manifest a minor degree of curvature. This fact may distort the perceived inheritance in the pedigrees used for linkage analysis. Indeed, from linkage studies with human pedigrees there is no concordance for identified loci.

3.6 Conclusion

To date, the underlying genetic cause of human IS has not been identified. The analysis of curveback guppies will suggest important genes involved in vertebral stability. In addition to a mapping cross, the curveback lineage will allow for a thorough investigation of curve inheritance through inbreeding and selection. Inbreeding of offspring from sibmate A and sibmate B has continued so that we presently have 15 families of fish enriched with genes that cause curvature. With a large pedigree we hope to correlate the segregation of curve types among families to genetic polymorphisms in candidate genes.

3.7 Acknowledgements

We acknowledge Julian Humphries and Tim Rowe of Digimorph, and support from Scoliosis Research Society and Natural Sciences and Engineering
Research Council of Canada. This research was approved by the SFU University Animal Care Committee, project #763B.

3.8 Literature Cited


11. *NIH Image.* Research Services Branch (RSB) of the National Institute of Mental Health (NIMH): National Institutes of Health (NIH), 2005


The *curveback* phenotype is a primary anterior lordosis (L) and a secondary kyphosis (K) occurring on the sagittal plane of the animal (A). Some individuals demonstrate coronal deviation (anterior axis: a; posterior axis: p) (B). Normal fish with sagittal plan and dorsal/ventral axes is shown (C). Digital photos of anesthetized adult females (A and C) taken on a standard light table under 3X magnification. Photo of female (B) is taken on an unanesthetized adult under 3X magnification.
Figure 3.2: Qualitative classification for curve types

Shown are 4 general classes of curvature used to score lordosis qualitatively. 

A: The severe, or progressed curve is referred to as type 4. 

B: Type 3 is considered a moderate curve. 

C: Type 2 is a slight curve. 

D: Nearly curved is type 1. All photos taken on a standard light table using anesthetized adult fish. Scale is in mm.
Figure 3.3: Anterior curve ratio

Quantitative measurement of the magnitude of lordosis (A), relative to the length of the fish [occipital orbit (OC) to caudal peduncle (CP)].
Figure 3.4: Computed tomography scan of curve apex

Vertebral detail shows no fusion or breaks. CT of apical vertebrae in curved (A) and normal (B) guppy. Scan consists of 350 0.4mm slices. Both guppies are of the curveback lineage. hs, indicates hemal spines; ns, neural spines.
Fish were scored from birth to 100 days after birth. Total number (#) of fish scored was 103; number of females was 54, and number of males was 49. Curve types are defined in Figure 2. Normal fish have a curve type '0'. Resolving curvature (type 5) is defined as a curve that has decreased by an order of 1 curve type or more.
Figure 3.6: Curveback pathogenesis

Curve progression during development is periodic, oscillating between periods of increase or decrease in magnitude and relative stasis. The range for the age of sexual maturity is indicated between the white lines. Male curve progression follows the same general trend as females.
As shown in Figure 3.7, the offspring of the sibmate A (F\textsubscript{3} progeny of the original cross) illustrate the complexity of curve magnitude inheritance. If inheritance were simple recessive, we would expect crosses \textit{ii} and \textit{iii} to have a high number of curved offspring, with no more than 50% in cross \textit{i}. In cross \textit{iii}, fish #28 resolved to normal, and the subsequent offspring were normal, while fish #22 and #26 progressed to type 4 at maturity.
Table 3.1 Parallels between human and guppy idiopathic spinal curvature

<table>
<thead>
<tr>
<th>DEVELOPMENTAL PARALLELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) No vertebral fusion or breaking.</td>
</tr>
<tr>
<td>2) Born normal, curve develops after birth.</td>
</tr>
<tr>
<td>3) Curvature does not hinder fitness of individuals.</td>
</tr>
<tr>
<td>4) Curve does not progress after skeletal maturity.</td>
</tr>
<tr>
<td>5) Curve magnitude increases with age.</td>
</tr>
<tr>
<td>6) Incidence of curve that resolves before maturity.</td>
</tr>
<tr>
<td>7) Onset time for curvature is variable.</td>
</tr>
<tr>
<td>8) Variability among individuals for rate and propensity of curve progression.</td>
</tr>
<tr>
<td>9) Female bias for most severe curve.</td>
</tr>
<tr>
<td>10) Angle growth is in spurts instead of linear progression.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GENETIC PARALLELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>11) Complex inheritance.</td>
</tr>
<tr>
<td>12) Major gene effect, but with additional multigenic modifiers.</td>
</tr>
<tr>
<td>13) Distribution of curve magnitude in population is continuous.</td>
</tr>
</tbody>
</table>
CHAPTER 4: IDIOPATHIC-TYPE SCOLIOSIS IS NOT EXCLUSIVE TO BIPEDALISM*

4.1 Abstract

Human familial/idiopathic-type scoliosis (IS) is a complex genetic disorder for which the cause is unknown. The curve phenotype characteristically demonstrates pronounced morphological and developmental variability that is likely a consequence of biomechanical, environmental, and genetic differences between individuals. In addition, risk factors that affect the propensity for curves to progress to severity are unknown. Progress in understanding the fundamental biology of idiopathic-type scoliosis has been limited by the lack of a genetic/developmental animal model. Prior to consideration of teleosts, developmental idiopathic-type scoliosis has been considered to be exclusive to humans. Consequently, there is the notion that the syndrome is a result of bipedalism, and many studies try to explain the deformity from this anthrocentric viewpoint. This perspective has been reinforced by the choice of animals used for study, in that chickens and bipedal rats and mice demonstrate idiopathic-type curvature when made melatonin deficient, but quadrupedal animals do not. Overlooked is the fact that teleosts also demonstrate curvature similar to chickens when made melatonin-deficient. Our characterization of the guppy curveback has demonstrated that non-induced idiopathic-type of curvature is not exclusive to humans, nor bipedalism. We hypothesize that unique morphological, developmental and genetic parallels between the human and guppy syndromes are due to common molecular pathways involved in the etiopathogenesis of both phenotypes. We explore established gene conservation between human and
teleost genomes that are in pathways hypothesized to be involved in the IS syndrome. We present non-induced vertebral wedging as a unique shared feature in IS and curveback that suggests a similar interaction between a molecular phenotype on the level of the vertebral anatomy, and biomechanics. We propose that rather than bipedalism per se, expression of idiopathic-type scoliosis is dependent on normal spinal loading applied along the cranio-caudal axis that interacts with an unknown factor causing the primary curve. In this regard, a comparative biological approach using a simplified teleost model will promote discovery of basic processes integral to idiopathic-type scoliosis in teleosts and humans, and highlight human-specific aspects of the deformity.

4.2 Introduction

Familial/idiopathic-type scoliosis (IS) is a complex genetic disorder that accounts for 80% of all human spinal curvatures (MIM 181800, Online Mendelian Inheritance in Man). It is broadly defined as a three-dimensional curve deformity with no known aetiology that manifests after birth and has a propensity to increase in magnitude with growth, until sexual maturity. Curve magnitude, morphology, rate of and propensity for progression are highly variable among individuals, due to biomechanical, environmental, developmental and possibly genetic variability between individuals.

An understanding of fundamental aspects of the deformity has been limited by the lack of a genetic/developmental animal model. Before consideration of teleost fishes, all observed forms of scoliosis in animals have been the result of congenital anomalies, or have been induced in laboratory
animals [1]. Hence, it appears that idiopathic-type spinal curvature is exclusive to humans, and therefore is alleged to be a consequence of bipedalism [1-6]. Abandoning this anthrocentric perspective will help advance our comprehension of not only the cause(s) of curve onset, but also risk factors associated with progression. A comparative biological approach using a simplified teleost model will promote discovery of basic processes integral to idiopathic-type scoliosis and highlight human specific aspects of the deformity.

4.3 How the choice of animal models for IS support the bipedal notion

That idiopathic-type scoliosis has never been observed in any animal other than humans certainly has encouraged the notion that the deformity is contingent on bipedalism. Experimentally, scoliosis has been produced using a variety of animals (i.e., rabbit, lamb, goat, mouse, rat, monkey, dog, pig, chicken). These all have the fundamental goal of producing a model that is comparable to IS in order to elucidate the aetiology, and promote new therapeutic methods [7]. Methods for induction of scoliosis include dietary deficiency, immobilization, local procedures (i.e. damage to spinal, neural or surrounding tissues), or pinealectomy. Ultimately, because they are induced, it remains controversial whether conclusions drawn from such experiments relate to primary or secondary influences for curvature [7,8].

Because most of the animals used for study of IS are quadrupedal, they have limitations for research into an aetiology that is presumed to be influenced by gravity [7]. The relevance of bipedalism and gravity to IS pathogenesis has
been supported by the fact that pinealectomy (or melatonin deficiency) can induce spinal curvature in chickens, but not in quadrupedal mammals unless they are forced to be bipedal [3,9-11]. For example, pinealectomized rats and mice made melatonin-deficient do not demonstrate spinal curvature as quadrupeds, but do if their front legs and tail are amputated in order to force them to be bipedal [9,10, 12].

Importantly, although never reviewed in orthopeadic studies, pinealectomy in the teleosts guppy and salmon induces spinal curvature with a physiological response similar to that in pinealectomized chickens [13-15]. Hence, the conviction that idiopathic-type scoliosis is exclusive to bipedalism and dependent on gravity has been biased by the selection of animals used for study.

### 4.3 Background on the curveback guppy

The *curveback* guppy is the first model for human IS to demonstrate spinal curvature in otherwise healthy fish that is not induced nor caused by congenital malformation of the vertebrae [16]. Our characterization of the guppy *curveback* syndrome has revealed unique morphological, developmental, and genetic parallels to human idiopathic-type scoliosis (IS).

The guppy is a small live-bearing teleost fish, and offspring are born approximately 3 weeks after conception. As with humans, the onset of curvature begins at variable ages after birth (guppy skeleton is completely ossified before birth) and can either stabilize at a moderate magnitude, resolve to normal or nearly normal, or progress to severity [16-18]. The curve phenotype is a primary sagittal lordosis of variable magnitude with most individuals exhibiting a posterior
kyphosis, coronal deviation and axial rotation (figure 4.1). Beyond complex inheritance, the human and curveback idiopathic-type curvature syndromes share: a female bias for severe curve magnitude, despite an equal incidence rate among males and females; similar variability for curve magnitude and morphology; variable age of curve onset and rate/propensity for progression; curve stabilization at sexual maturity; the incidence of resolving curves; and vertebral shape distortion at the apex of severe curves [16].

4.4 Hypothesis

Study of the teleost curveback provides an important insight: that idiopathic-type scoliosis is not a human exclusive deformity. Here we explore the hypothesis that common molecular pathways are involved in the etiopathogenesis of the guppy and human phenotypes. This idea is based on the fact that curveback demonstrates so many phenotypic parallels to IS, and that humans and teleosts share many genes involved in basic biological processes. It is possible that the same genes in human and guppy idiopathic-type scoliosis are mutated, or it is also possible that different sets of genes are mutated in guppy and human systems, but that they affect common molecular pathways. Either way, comparison of the two systems has the potential to illuminate important biological pathways involved in the maintenance of spinal stability throughout growth.

An important corollary of our hypothesis is that rather than a consequence of gravity and bipedalism per-se, the deformity is likely contingent on the interaction of force/loading applied along the cranio-caudal axis with the vertebral
anatomy, in the presence of a genetic predisposition. An important question that emerges from our hypothesis is whether genes for idiopathic-type curvature are present in terrestrial animals, but their expression is constrained by quadrupedal biomechanics, or if indeed the primary aetiology is exclusive to humans and teleosts.

4.5 Is the genetic predisposition exclusive to humans?

It is possible that the genetic predisposition and components related to curve progression for idiopathic-type curvature in guppies and humans are in the same genes or in genes controlling common genetic pathway(s). The observed phenotypic variation and lack of concordant loci identified among human linkage studies has suggested that there may be multiple predisposing genes for IS [19-22]. With complex syndromes such as IS, different polymorphisms in the same gene or in different genes within the same molecular pathway could cause observed phenotypic variability [23-26].

Fish share most developmental pathways, physiological mechanisms and organ systems with humans [reviewed in 27-29]. Comparisons between human and fish genomes have identified DNA sequences and entire gene networks that have significant functional activity in humans, many of which are in systems implicated for human IS, suggesting some gene conservation for genetic factors thought to be involved in the deformity (e.g. osteoblast and chondrocyte differentiation [30], bone formation [31,32], muscle formation [33], gene regulation [34], pineal gland (Lhx9) [35], neural development [36], somitogenesis [37], cell proliferation [38-40], pituitary(pitx) function [41], osteoclast function [42]). These
include regulation of hormones that might be involved in idiopathic-type curvature (based on suggestions from human studies): e.g. calmodulin and steroidogenesis [43], nutritional regulation of growth hormone and insulin-like growth factor-I [44], IGF-binding proteins (IGFBPs) [45], growth hormone-releasing hormones and receptors [46], a neuromodulatory role for nitric oxide [47], thyroid hormones (TH) in bone remodelling [48], thyroid and muscle growth [49], the midbrain locomotor region (MLR) and descending (reticulospinal) pathways that activate spinal networks for rhythmic movements such as swimming in fishes, and walking and running in humans [50,51].

4.6 Physical evidence for shared biomechanical and/or physiological factors

Distortion of vertebrae at the apex of curvature is considered an important component of the human phenotype that has not been observed in other animals unless it is induced. It is broadly suggestive of an unknown physiological dysfunction involving asymmetrical loading (the details of which are a subject of speculation) the spine during growth that directly affects the vertebral body growth plates, so that longitudinal growth of a vertebral body is modified [reviewed in 52]. Because the non-induced phenotype is (without consideration of teleosts) exclusively human, it has generated many hypotheses that are difficult to test [reviewed in 53]. Symptomatic distortion of apical vertebrae may provide valuable perspective regarding the interaction between a molecular phenotype on the level of the vertebrae and biomechanics. Therefore, study of vertebral distortion has involved simulation of the phenotype by bracing in rat and cow tails.
or tethering in goats or rabbits [54-58]. Importantly, the question of to what extent curvature is due to altered biomechanics and growth and how much is due to a more primary aetiology cannot be answered only using physical simulations in induced models.

In both humans and guppies primary loading on the spine is along the cranial-caudal axis. In simplified terms, normal loading on human vertebrae is from the weight of the head and gravity coupled with the loading associated with bipedalism (i.e. standing and walking); in guppies, from swimming through the dense medium of water coupled with the force associated with the tail-beat motion. In guppies, non-induced distortion of apical vertebrae is similar to that observed in human IS, in which vertebral bodies are compressed on the concave side of a curve [16, 59-61]. An important question in both the curveback and human phenotypes is whether the vertebral bodies are compromised so that they are less capable of handling normal cranio-caudal loading (i.e., failure of mechanotransduction), or if the vertebral bodies are normal, but there is excessive/pathological force on the vertebrae sufficient to cause distortion (i.e., dysfunctional growth). There are hypotheses to support the idea that the predisposing defect may involve vertebral bodies [62-65], and also there are hypotheses to support that there may be excessive force on the vertebrae from growth related dysfunctions [66-68].

4.7 Consequences of hypothesis

One of the main insights of the curveback model is that idiopathic-type scoliosis is not exclusive to bipedalism. We hope that such a consideration will
provoke new ideas regarding which components of the syndrome are primary or initiating, and which are secondary (complicating, or risk factors associated with the propensity for curve progression), and how these factors might interact. Human and guppy biomechanical similarities might elucidate essential components of idiopathic-type curvature, and differences between the two animals may offer the opportunity to specify which aspects of IS are indeed exclusive to humans.

Comparative studies of guppy and human physiology and curve phenotypes might direct hypotheses regarding how biomechanics can interact with intrinsic aspects of curve aetiology (i.e. genetic and molecular aspects) and/or progression (i.e. growth related aspects). Hypotheses regarding the relative contribution of factors such as tallness/length [69-73], dorsal shear force [1], pelvic association [74-76], or posture [77-81] can be critically evaluated by comparison to the anatomy of curveback.

With complex human syndromes that involve interactions among genetic, physiological, and environmental forces, a successful experimental approach is to first identify genes and molecular pathways in a model animal with a similar phenotype [82-85], and we have presented one for IS, the guppy curveback. Once genes involved in the aetiology of curveback are identified, we can determine whether mutations in these genes are correlated to the human IS phenotype. An important question that then can be answered is whether the genetic predisposition to idiopathic-type curvature is common to all vertebrates
but not expressed in quadrupeds because of biomechanical constraints, or if unique mutations in the human lineage have lead to this prevalent syndrome.

4.8 Acknowledgements

Support from Natural Sciences and Engineering Research Council of Canada; and Award Number R21AR053730 from the National Institute Of Arthritis And Musculoskeletal And Skin Diseases.

4.9 Literature Cited


[43] Benninghoff AD, Thomas P. Involvement of calcium and calmodulin in the regulation of ovarian steroidogenesis in Atlantic croaker (Micropogonias
undulates) and modulation by Aroclor 1254. *General and Comparative Endocrinology* 2005;144: 211-223.


Figure 4.1

A and B: Sagittal profile of a normal (A) and curved (B) adult curveback guppy. C: Coronal profile of the same female as shown in B. Photos taken on euthanized fish with digital camera (Kodak Easyshare Z612) under 3X magnification on a light table. Scale shown is mm.
CHAPTER 5: MORPHOLOGICAL CHANGES ASSOCIATED WITH VERTEBRAE INVOLVED IN IDIOPATHIC SPINAL CURVATURE IN THE GUPPY
5.1 Abstract

We investigate morphological change associated with the vertebrae of otherwise healthy guppies with idiopathic spinal curvature. Analysis of whole-mount skeletal and histological specimens were used to 1) characterize the morphology of normal guppy vertebrae, 2) investigate structural and microarchitectural changes in the vertebrae of guppies with spinal curvature, and 3) compare the guppy vertebral changes to known data from mammalian models with induced curvature, other teleost species with spinal curvature, and human idiopathic scoliosis (IS). Despite ontological differences, the guppy structural modifications are similar to those described for human IS and induced mammalian models. Microarchitectural changes are comparative to that of other teleosts with acellular bone. We suggest that the morphological changes in guppy vertebrae are an adaptive response to unknown force acting along the cranial-caudal axis of the spine.

5.2 Introduction

The guppy, *Poecilia reticulata*, is an advanced teleost that is closely related to the medaka, *Oryzias latipes*. A supertree phylogeny by Mank et al. (2005) places beloniformes (medaka) and cyprinodontiformes (guppy) as sister orders in the superorder Acanthopterygii. As such, the guppy is composed of acellular (anosteocytic) bone. This is in contrast to basal teleosts (such as the zebrafish or salmon) that, as with mammals, have vertebrae composed of cellular bone that contains osteocytes embedded in the bony matrix and is derived from endochondral ossification. The acellular bone of advanced teleosts (such as the
guppy, medaka, and sea bass) is devoid of embedded osteocytes, and the vertebral centra have no cartilaginous precursor (they are formed by intramembranous ossification) (reviewed in Witten and Huysseune, 2009).

The curveback lineage of guppy is characterized by heritable idiopathic spinal curvature (i.e. curvature is not explained by fused or broken or malformed vertebrae) that develops during growth of otherwise healthy fish. Although inbreeding has confirmed a genetic basis, the aetiology for spinal curvature in curveback is unknown. The phenotype is characterized as a sagittal anterior lordosis and posterior kyphosis, occurring in the tail (i.e. vertebrae posterior to the vent and not associated with ribs) (Fig. 5.1). For clarity, the portion of the body anterior to the vent is referred to as the trunk (this includes the head), and the portion of the body posterior to the vent is referred to as the tail. Fish are born with normal spines (guppies are live-bearing fish) and develop curvature as they grow. Offspring are born with a fully ossified skeleton after ~ 3 weeks of gestation, and sexual maturity is at about one month past birth. The curves generally do not progress once sexual maturity is reached, and the final adult curve magnitude is variable among individuals (see Gorman, et al., 2007).

Whole-mount staining of juvenile fish has demonstrated that vertebral bodies are normal at curve onset and in curves with mild magnitude (Gorman and Breden, data unpublished). In curves of higher magnitude, the amphicoelous shape of vertebrae is distorted. In this study, we use whole-mount skeletal and histological analyses to investigate structural and micro-architectural changes associated with the tail vertebrae in the curveback guppy. We relate these
findings to what is known from prior studies in teleosts, and mammals with a similar vertebral phenotype.

5.3 Materials and Methods

The guppy is a small, live-bearing teleost native to the streams of northeast South America, which has been a model organism for ecological, evolutionary, developmental, and genetic research since the 1920’s. The curveback lineage was established from laboratory guppies that are derived from a wild population caught in Central Cumaná, Venezuela and raised under standardized conditions since 2000. The lineage originated from a curved male crossed to a normal, unrelated female in 2003, followed by full-sib mating and backcrossing. Laboratory fish are kept under standardized conditions [i.e., fed with flakes (Nutrafin, www.hagen.com) or brine shrimp nauplii, alternately every afternoon; 25-26° C ambient temperature; pH 7-9; RO water reconstituted to 1600-1800 ppm salinity with aquarium salts; 14/10 hour light/dark cycle], in compliance with protocols approved by the Simon Fraser University animal care facility and the Canadian Council on Animal Care.

Sample preparation:

The adult males and females who were chosen for whole-mount skeletal analysis were matched for the magnitude of their curves, based on qualitative scores defined in Gorman et al. (2007). All curves in this study were of a qualitative score 2 or 3, where 0 represents non-curved and 4 represents the greatest magnitude. All fish were euthanized at a minimum of 3 months past birth
and photographed on a light table with a digital camera (Toshiba PDR-3310, NYC, USA) under 3X magnification before being fixed in 10% neutral-buffered formalin (Sigma).

**Whole-mount skeletal staining:**

Twenty-seven adult fish (14 females: 6 curved, 8 non-curved; 13 males: 6 curved, 7 non-curved) were stained for bone and cartilage. Specimens were fixed for 2 days, and then washed in several changes of ddH$_2$O, (>1 hour each), then dehydrated in 50%EtOH (24 h) and then 100% EtOH (24 h). Whole fish were then incubated in cartilage staining solution (8GX Alcian blue in 70% anhydrous EtOH and 30% acetic acid) for 24 hours at room temperature with mild agitation. Stained specimens were neutralized in saturated aqueous sodium borate solution for 9-12 hours and then bleached for 20 minutes (3% hydrogen peroxide and 1% potassium hydroxide). Specimens were then digested with a trypsin solution (1g trypsin in 35 ml saturated sodium borate/ 65ml ddH$_2$O) until they were 60% clear. Bones were stained with Alizarin red solution in 0.1% potassium hydroxide solution at room temperature with mild agitation, until they were light red in colour. Specimens were destained in trypsin for 40-48 hours and then preserved in stepwise solutions of 30%glycerol/70% of 1% KOH, 60% glycerol, and 100% glycerol. Thymol was used in preserved specimens to inhibit contamination. Stained specimens were viewed through a Meiji MEI (Tokyo, Japan) stereoscope under 4X and 2X magnification, and photographs were taken with a Pentax MX camera though a MA150/T2 (Meiji) adapter.
Histology:

Tails from four curved and two non-curved curveback females were fixed overnight in 10% neutral-buffered formalin and then demineralized in a solution of 10% formaldehyde: 5% ethylenediaminetetraacetic acid (EDTA) for a minimum of two weeks. For half of the curveback specimens, tail vertebrae 4 and 11, which occur at the apex of the lordosis and kyphosis, respectively, were carefully dissected out under a stereomicroscope. This was to ensure that they could later be precisely embedded for sectioning in the transverse. Tails to be sectioned in the sagittal plane were left intact.

Following several rinses in 1X phosphate-buffered saline (PBS), body segments were dehydrated through an ethanol:PBS series, cleared in xylene, and penetrated with paraffin under vacuum. Specimens were embedded under a stereomicroscope and sectioned in either the transverse or sagittal planes at a thickness of 7-10 μm. Optimal sectioning generally required that the blocks were first ‘softened’ by soaking in distilled water.

Tissue sections were stained with either Mallory’s trichrome (as per Handrigan and Wassersug, 2007) or double-stained with Picrosirius red and Alcian blue (as per Ashique et al. 2002). By the former protocol, collagen fibrils are stained blue. By the latter, collagen type I and bone appear red and sulphated proteoglycans, which are enriched in cartilage, as blue. Sections were photographed using a Hitachi HV-F22 3-CCD digital camera coupled to a Zeiss Axioskop compound microscope.
Analysis:

Because curvature is manifested only in the tail, our analysis is limited to the vertebrae therein. To quantify vertebral distortion associated with curveback independent of individual size differences, we calculated five ratios from seven measured lengths on each vertebral body for curved and non-curved males and females (table 5.1). For each vertebra, all five ratios were compared between: 1) non-curved females versus non-curved males; 2) curved females versus curved males; 3) curved males versus non-curved males; 4) curved females versus non-curved females. A two-tailed Student’s t-test was used to compare group means, and Levene’s test was used to compare variability among groups. A sequential Bonferroni correction was employed for each test where \( \alpha=0.0025 \), number of tests was 20 (4 comparisons for five ratios) (Rice, 1989). The structure of each vertebra was considered a separate question (a total of 15 vertebrae were considered for each individual). Measurements were taken on digitized photos that were taken under 2X magnification (ImageJ software, http://rsbweb.nih.gov/ij/). Statistical analysis was conducted using JMP software for Mac, Version 7.0 (SAS Institute, INC., Cary, NC, USA).

5.4 Results

Characterization of normal guppy, Poecilia reticulata, vertebrae

Normal structural morphology

All curved and non-curved whole-mount specimens of curveback have 15 vertebrae in the tail portion of their body. The first vertebra of the tail is identified as the anterior-most vertebra that is not associated with ribs, and the last tail
vertebra is located before the urostyle of the caudal complex (Fig. 5.2). With the exception of the first two vertebrae, the normal vertebral column of the tail is a metameric structure with each vertebra comprising an amphicoelous (hour-glass shape) centrum, with paired neural arches on its dorsal face and haemal arches ventrally. Among teleosts, the last three vertebrae in the tail (numbers 13, 14, 15) are associated with the caudal complex and are sometimes referred to as preural (Fujita, 1992; Faustino and Power, 1998; Koumoundouros, et al., 1999). The neural and hemal spines of these preural vertebrae are fixed to the caudal complex by cartilage (apparent in Fig. 5.2). Among the family Poeciliidae, the anal fin and vertebrae involved in its suspension are sexually dimorphic, so that in mature males, the anal fin is transformed into the gonopodium and the first two caudal vertebrae form its suspensorium (see Fig. 5.2) (Rosen and Bailey, 1963). Among non-curved males, the first tail vertebra has a significantly thicker midline width than that of non-curved females (thickness ratio/ comparison 1). This difference between genders is likely related to physiological and biomechanical factors associated with the male gonopodium and its suspensorium.

Normal vertebral microanatomy of the guppy

Histological examination reveals that the axial skeleton of the guppy comprises a series of amphicoelous vertebral centra coupled by non-cartilaginous intervertebral ligaments (Fig. 5.3). Vertebral centra consist of acellular or osteoid bone, which lacks occluded osteocytes and is enriched with types I and III collagen, as revealed by staining with Picrosirius Red and Mallory's trichrome (Fig. 5.3B-G). Vertebrae were negative for Alcian Blue staining (Fig. 5.3D-G),
indicating that they lack sulphated proteoglycans, a primary constituent of cartilage. This is consistent with most other advanced teleost species, which form vertebral body bone without a cartilage intermediate (Francois, 1966; Laerm, 1976; Grotmol et al., 2003; Kranenbarg, et al., 2005a; Nordvik et al., 2005; Inohaya et al., 2007; Witten and Huysseune, 2009). Vertebral centra encompass the remnants of the embryonic notochord (Francois, 1966; Laerm, 1976; Schmitz, 1995; 1998). This structure is highly vacuolated, particularly at the middle of the vertebral body, where it forms a single, great lumen enclosed by a thin outer sheath of fibrous tissue. At intervertebral levels, however, the notochord is considerably less vacuolated and more fibrous in composition.

The intervertebral ligament of *P. reticulata* is built from the notochord and a ring-like structure that rests directly between adjacent vertebral centra. For simplicity, we refer to these two structures by the name of the analogous intervertebral components in humans. By adopting the human terminology for guppy intervertebral ligament components, we are not implying that the structures represent *de facto* evolutionary homologs or are identical in composition. Rather, we are highlighting similarities in overall organization of the ligament and the likely function of each of its components. In humans, the intervertebral disc contains a tough outer cortex, the annulus fibrosus, and a spongy medullary layer, the nucleus pulposus. Thus, to refer to the outer ring-like structure of the guppy intervertebral ligament we use the term 'annulus fibrosus.' In humans, this organ comprises fibrocartilage and serves to distribute forces evenly around the disc. The fibrous appearance and enrichment for sulphated proteoglycans of the
guppy annulus fibrosus suggests that it is firm and may function similarly as in humans.

We term the vacuolated inner core of the guppy intervertebral ligament the ‘nucleus pulposus.’ We further subdivide the structure into an outer ‘spongy’ layer and an inner ‘fibrous’ layer (Fig. 5.3C). In humans, the nucleus pulposus comprises chondrocytes, proteoglycans and collagen and functions as the shock absorber for the disc. While the guppy structure lacks cartilage altogether, staining negative for Alcian Blue (Fig. 5.3D-G), its highly vacuolated character also points to a role for this structure in shock absorbance.

**Morphological changes associated with spinal curvature**

**Change in vertebral shape**

With lordosis, the concave side of the curve is dorsally directed, and with kyphosis the concavity is ventrally directed. Vertebrae involved in curvature typically display distortion of the amphicoelous shape that appears to be most extreme at the apex of curvature, and generally more pronounced in curves of greater magnitude *(Fig. 5.4)*. Analysis of height ratios reflects that there is generally a greater height on the concave side of a vertebral body coupled with a reduced height on the convex side, suggestive of anterior-posterior compression. Analysis of length ratios shows that there is no dorsal-ventral distortion in affected vertebrae. The vertebral arches appear to be distorted for the vertebrae located at the apex or curvature. Although there seems to be some morphological changes in the arches of vertebrae associated with curvature, here we characterize changes observed in the vertebral centra.
Significant differences between males and females, and curved and non-curved individuals for either mean values or variability of vertebral ratios are summarized in table 5.2 and Fig 5.5. After correction for multiple comparisons, the vertebrae that are significantly affected by lordosis are numbers 2 and 4 in females (height ratio/ comparison 4: curved females vs. non-curved females), and number 1 in males (slenderness ratio/ comparison 3: curved males vs. non-curved males). Those vertebrae most affected by kyphosis are 11 in females (height ratio/ comparison 4), and 12 in males (thickness ratio/ comparison 3). In females, tail vertebra 8 shows a significantly reduced relative anterior width. This vertebra is not usually involved in lordosis or kyphosis, but rather is situated at the transition between curves (i.e., the point of inflection). Among curved female guppies, a significantly reduced anterior width for vertebra 8 suggests that although not involved in lordosis or kyphosis, it is likely modified by these curves.

Although the males and females selected for whole-mount analysis were matched for curve magnitude, vertebral ratios reflect slight morphological differences in the overall structure of their curves. The ratio that best reflects vertebral change associated with curvature in females is the height ratio, while curvature in males is represented by ratios that are associated with the thickness of vertebrae (slenderness and thickness ratios). When curved males and females were compared to each other, vertebral height in females shows significantly more variable ratios for vertebra 4 (height ratio/ comparison 2: curved females vs. curved males), which appears to be the apex in most female lordotic curves. In curved males, the first tail vertebra is less slender overall than that of
non-curved males (slenderness ratio/ test 3), and vertebra 5 is less slender (or more compressed) compared to females (slenderness ratio/ comparison 2).

**Micro-anatomical defects in vertebral morphology and inter vertebral ligament orientation**

Defects in both vertebral morphology and intervertebral orientation were observed in *curveback* guppies (Fig. 5.6A,E) as compared to non-curved (Fig. 5.6B). Shape distortion was noted primarily on the side of the vertebra facing the concavity of the spinal curvature. Thus, lordotic vertebrae primarily display defects dorsally (Fig. 5.6A) and kyphotic vertebrae are affected on their ventral surface (Fig. 5.6E). In the transverse plane of section, vertebral centra appear slightly thicker; vertebral arches are more visibly affected, appearing either bilaterally asymmetric or else broadened at their base (Fig. 5.6A,E). In the longitudinal plane, scoliotic vertebral centra appear bent in shape (Fig. 5.6B,F).

The most pronounced defects associated with idiopathic curvature in the *curveback* guppy are in intervertebral ligaments occurring at the apex of spinal curvature (Fig. 5.6B,F). For these ligaments, the fibrous nucleus pulposus appears to migrate towards the convex face of the spinal curvature in response to compressive forces acting on the concave side of the ligament (Fig. 5.6B',F'). This displacement of the fibrous core causes compression of the spongy nucleus pulposus on the convex face and distension on the opposite side. Thus, in intervertebral discs occurring at the apex of a lordotic curve, the fibrous nucleus pulposus migrates ventrally, while the spongy cortex becomes distended dorsally (Fig. 5.6B,B'). Conversely, in kyphotic curves, the fibrous core of the nucleus
pulposus is displaced dorsally and the spongy outer layer distended ventrally (Fig. 5.6F,F').

5.5 Discussion

We performed whole-mount skeletal staining on 27 (12 curved and 15 non-curved) adult male and female guppies from the *curveback* lineage in order to characterize the normal vertebral morphology as well as changes associated with non-induced (hereditary) spinal curvature. Although spinal curvature among teleosts is common, (reviewed in Gorman and Breden, 2007), the majority of studies are conducted on animals with obvious defects such as broken or malformed vertebrae. Because no other teleost study has described vertebral distortion similar to that observed in *curveback*, we draw our comparisons regarding the changes in vertebral shape from mammalian studies. With regard to microarchitectural changes however, there are some interesting teleost studies to draw from.

Changes in vertebral shape that are associated with spinal curvature

To characterize changes in vertebral shape, we and tested for differences between normal and curved individuals and between genders using 5 ratios for each vertebra. Vertebrae with significant differences are shown in table 5.2 and Fig 5.5. Generally, we found that for the vertebrae of curved guppies, the normal amphicoelous (hour-glass) shape of the centra are distorted so that vertebral height is reduced on the convex side of curvature relative to the concave. Vertebral distortion is generally more pronounced at what appears to be the apex.
of curvature (for lordosis and kyphosis). This effect is significantly pronounced among females, while among males vertebrae were significantly more compressed. Slight differences between male and female curve morphologies could be due to physiological factors related to the gonopodium and its suspensorium. Although curvature does not affect the number of offspring born per female in the curveback population, it is unknown whether males have adopted compensatory behaviours in their breeding/courtship because of curvature. For example, increased gonopodial flicking (a typical courtship behaviour) could increase or change the force applied to the tail vertebrae and might explain why in curved males vertebrae 1 and 5 become thicker compared to non-curved males.

The distortion of vertebral shape associated with idiopathic spinal curvature in the guppy resembles that described for human idiopathic scoliosis (IS). In the human phenotype, the normal vertebral body shape at and around the apex of curvature becomes characteristically distorted into a wedge (defined by a reduction in vertebral length on the concave side of a curve relative to the convex side) (Stokes and Aronsson, 2001; Beuerlein, et al., 2003; Majcher, et al., 2003; Parent, et al., 2004; Parent, et al., 2005). This wedging of the vertebral centra in IS patients is often explained by the Hueter-Volkmann law, which states that vertebral body growth is retarded by continuous or excessive mechanical compression upon the epiphysis (the cartilaginous growth plate), resulting in a reduction of vertebral length (Stokes, et al., 1996; 2006). With regard to IS, asymmetrical compression upon the epiphysis is hypothesized to cause the
observed reduction in vertebral length on one side of a vertebra. The source of this asymmetrical compression is unknown. Several authors assert that the Hueter-Volkmann law explains the progression of curvature in IS by a 'vicious cycle' in which asymmetric vertebral growth induces the vertebral wedge shape that in turn promotes asymmetric spinal loading along the cranial-caudal axis, and encourages further asymmetric growth, greater shape distortion, and a higher curve magnitude (McCarroll and Costen, 1960; Stillwell, 1962; Dickson, et al., 1984; Mente, et al., 1997; Machida, 1999; Stokes, et al., 2006). However, there is a lack of evidence demonstrating that the Hueter-Volkmann law contributes to biomechanical effects on vertebral body growth rates in patients with IS (Castro, 2003).

Humans are the only mammals to demonstrate heritable idiopathic spinal curvature. The occurrence of non-induced spinal curvature in mammals is often associated with fused, broken, or malformed vertebrae. Nevertheless, distortion of vertebral shape consistent with the Hueter-Volkmann law has been demonstrated experimentally in mammalian models with induced curvature (Stilwell, 1962; McCarroll and Costen, 1960; Aronsson, 1999; Mente, et al., 1997; Mente, et al., 1999; Kin, 1994; Braun, et al., 2003; 2006a; 2006b). With induced models, asymmetrically loaded vertebrae demonstrate changes in growth rate along their growth plates, causing uneven progression in longitudinal growth and consequential shape distortion in the form of wedging similar to that observed in human IS (Mente, et al., 1997).
The vertebral column of the guppy is formed via intramembranous ossification and so cartilage that would account for the Hueter-Volkmann law is not present (Mookerjee, et al., 1940). This fact likely explains why there is not a relative reduction in the dorsal concave or ventral concave vertebral lengths of lordosis or kyphosis, respectively (as measured by the width ratio), and suggests that longitudinal growth of the vertebrae is not affected by curvature. The fact that the concave sides of affected guppy vertebrae are distorted suggests that as in mammals, asymmetric force is applied along the cranial-caudal axis of the spine. However, mechanisms that explain the apparent shape distortion associated with this force are appear to be different in mammals and the guppy.

**Microarchitectural changes in the vertebrae of curved guppies**

Idiopathic curvature in guppies is associated with micro-anatomical changes in both the vertebrae and intervertebral ligaments (Fig. 5.6). Centra and vertebral arch bases appear thicker on the side of vertebrae facing the concavity of a curve. We attribute these changes to either (1) distortion of normal vertebral shape or (2) active remodelling of vertebral osteoid bone in the face of extrinsic forces associated with curvature. The latter phenomenon has already been described in a number of teleost species (Laerm, 1976; Glowacki et al, 1986; Witten and Villwock, 1997; Huysseune, 2000; Kranenbarg et al., 2005a; 2005b). Most recently, Kranenbarg and others (2005a, 2005b) described remodelling of lordotic acellular vertebrae in the sea bass, Dicentrarchus labrax. The authors convincingly demonstrated by volumetric analysis that lordotic vertebrae underwent adaptive bone formation in response to altered axial loading.
associated with curvature. A similar approach should be taken to determine whether curveback guppy vertebrae are undergoing active osteoid remodelling. For now, corroborative support for the phenomenon in curved guppies comes from our observation that defects occur predominantly at the articular surface of vertebrae (Fig. 5.6), the 'growth zone' for centra. New bone matrix is laid down here by osteoblasts housed in the intervertebral ligament and originally derived from the embryonic sclerotome (Inohaya et al., 2007).

There is limited experimental study performed on bone from patients with IS, because of the lack of available of tissue (Parent, et al., 2004). Therefore, adaptive vertebral changes have been studied indirectly by either inducing curvature in mammalian models or in structures related to vertebrae such as the vertebral facets of IS patients. Mammalian models with induced spinal curvature have shown that cortical thickness increases and porosity decreases in areas of mechanical compression, while cortical thickness decreases and porosity increases with tension (Skedros, et al. 1994; 1996; Su, et al., 1999). Histological study of adaptive changes in facet micro-architecture from 8 IS patients demonstrated remodelling similar to that observed in animal models with induced curvature, where bone is subject to known eccentric tension-compression forces (Lanyon and Rubin, 1984; Shea, et al., 2004). Also, in scanning electron microscopy performed on spinal facets of patients with IS, there is a thicker cortex on the concave side of a curve (suggesting excessive force) and more porous bone on the convex side (suggesting tension forces) (Shea, et al., 2004).
Driscoll and others (2009) investigated the health of the intervertebral disc, migration of the nucleus pulposus, and trabecular bone mineral density (BMD) as indicators of concave-convex stresses associated with curvature in IS. In a finite element model, they demonstrated that increased BMD, annular degeneration, and disc migration played a moderate role in the progression of curvature because they altered the path of force transmission within the spine. In curved guppies the nucleus pulposus is displaced towards the convexity of the curve (Fig. 5.6B,F) (in both lordosis and kyphosis), closely mirroring intervertebral disc migration associated with IS (Ponseti et al., 1976; Violas et al., 2005; Driscoll, et al., 2009). This convergence of phenotypes suggests that, despite significant differences in histological composition, the human and guppy intervertebral ligaments are functional homologs of each other and are affected by qualitatively similar extrinsic forces resulting from curvature. The fact that in both human and guppy idiopathic curves defects predominantly occur at the articular surface of vertebrae (i.e. the ‘growth zone’ for centra), suggests that biomechanics associated with cranio-caudal loading are an important consideration in the overall phenotypes.

5.6 Conclusions

Distortion of vertebral shape and micro-architectural changes in guppy idiopathic spinal curvature indicates: (1) asymmetric spinal loading along the cranio-caudal axis, and (2) greater compressive force on the concave side of curvature. Despite a lack of cartilage that has explained mammalian vertebral shape distortion in response to asymmetric spinal loading, morphological change
guppy vertebrae indicate similar concave-convex biases as human idiopathic scoliosis. The skeletons of teleost fish and mammals are both subject to adaptive remodelling in response to mechanical stress. Experiments with mammals and teleosts describe an adaptive response that is similar to that seen in the *curveback* guppy (Shea, *et al.*, 2004; Kranenberg, *et al.*, 2005a; 2005b). Therefore, distortion of vertebral shape, vertebral thickening, and intervertebral disc migration in the guppy idiopathic spinal curvature are likely adaptive responses and not causes for curvature.

5.7 Acknowledgements

This study received support from the Natural Sciences and Engineering Research Council of Canada, and the Scoliosis Research Society. We are thankful to Dr.s Wainwright and Parichy for their clear and stain protocols, which we adapted for guppies. We are thankful to Dr. S. Tredwell for his input and interest.

5.8 Literature cited


Su SC, Skedros JG, Bachus KN, Bloebaum RD, 1999. Loading conditions and


Figure 5.1: The *curveback* phenotype

A. Non-curved adult female from the with trunk and tail portions of the body defined by being either anterior or posterior to the vent.

B. The *curveback* phenotype is defined by a dorsal-ventral deviation of the spine. Specifically, there is an anterior lordosis and a posterior kyphosis. Adult females photographed with under 3X magnification with a digital camera on a light table. Scale shown in mm.
Figure 5.2: The Caudal Skeleton

The first (1) and last (15) tail vertebrae, shown in an adult female. Cartilage that fixes the last three tail (preural) vertebrae to the caudal complex is stained with Alcian blue (arrow). Insert shows the haemal spines of first two vertebrae of an adult male that are transformed into the suspensorium for the gonopodium (g). Scale bar is 1mm.
Figure 5.3: Normal vertebral and intervertebral microanatomy of the guppy, *Poecilia reticulata.*
(A) Whole-mount skeletal preparation of tail vertebrae shown in lateral view. Vertebral centra in *P. reticulata* are amphicoelous and bear paired arches dorsally and ventrally. (B-G) Micrographs of guppy vertebral sections stained by either Mallory's trichrome for collagen (B,C) or with a combination of Picrosirius red and Alcian blue (D-G), which stain for collagen and sulphated proteoglycans, respectively. (B) Sagittal section through vertebral column, showing three successive inter-vertebral ligaments (visible in whole-mount in boxed area in panel A). (C) Magnified view of boxed area in B. The intervertebral ligament of *P. reticulata* comprises two structures: an outer ring-like structure ('annulus fibrosus') and a spongy, notochord-derived tissue ('nucleus pulposus') that forms the core of the ligament and runs between adjacent centra. For clarity, these structures have been digitally colorized in panel C'. The nucleus pulposus stains strongly for collagen, while the annulus fibrosus is enriched for sulphated proteoglycans (arrowhead in inset). (D-G) Consecutive transverse sections through a single vertebra, spanning the inter-vertebral region to the tapered middle of the centrum (see vertical lines in panel B for approximate axial level). Remnants of the embryonic notochord line the inside of the vertebral centrum and, at intervertebral levels, form a wall between vertebrae. Inset in panel D depicts a schematized intervertebral ligament in transverse section; colours correspond to those shown in panel C'. Abbreviations: ao, aorta; ha, hemal arch, m, axial muscle; na, neural arch; sc, spinal cord, v, vacuole; vc, vertebral centrum. Scale bar equals 200 μm.
Figure 5.4: Vertebral shape distortion associated with curvature

**A:** Normal vertebrae in adult female [centrum (c), neural arch (na), and hemal arch (ha)].

**B:** Lordotic vertebrae in adult *curveback* female: distortion of the amphicoelous shape is reflected by a reduced ventral height and increased dorsal height.
Tail vertebrae most significantly affected by curvature in females are shown as the affected vertebral number in circles; vertebrae affected in males are shown as squares. See table 2 for significant values.
Figure 5.6: Vertebral and intervertebral defects associated with spinal curvature in the *curveback* guppy.
Histological sections through normal and scoliotic vertebrae are presented for side-by-side comparison. Sections were cut in both the transverse and sagittal planes and stained with a combination of Picosirius red and Alcian blue, which label collagen and sulphated proteoglycans, respectively. (A,C,E) Compared to normal vertebrae, lordotic and kyphotic vertebrae appear thicker and asymmetric in shape (white arrowheads) on the concave face of the spinal curve. This suggests that scoliotic vertebrae are being distorted or actively remodelled in response to biomechanical stress associated with curvature. (B,D,F) Within the intervertebral ligament of both lordotic and kyphotic vertebrae, the fibrous portion of the nucleus pulposus appears to migrate towards the convex face of the spinal curvature. The white arrow in panel B’ and F’ indicates the direction of this migration. The displacement of the ‘fibrous nucleus pulposus’ causes compression of the ‘spongy’ portion on the convex face and distension on the opposite side (dotted outlines). Abbreviations: ao, aorta; ha, hemal arch; m, axial muscle; na, neural arch; v, vacuole; vc, vertebral centrum; sc, spinal cord. Scale bar equals 250μm.
Table 5.1: Five ratios used to characterize vertebral distortion

<table>
<thead>
<tr>
<th>NAME OF MEASUREMENT</th>
<th>DEFINITION</th>
<th>Diagram of lengths used for ratios</th>
<th>PURPOSE</th>
</tr>
</thead>
</table>
| Length Ratio        | Dorsal length/ Ventral length  
Length of line 1/ Length of line 2 | ![Diagram](image1) | Wedging along vertebral length |
| Width Ratio         | Anterior width/ Posterior width  
Length of line 3/ Length of line 4 | ![Diagram](image2) | Wedging along vertebral width |
| Height Ratio        | Dorsal height/ Ventral height  
Length of line 5/ Length of line 6 | ![Diagram](image3) | Distortion of amphicoelous shape |
| Thickness Ratio     | Midline width/ Posterior width  
Length of line 7/ Length of line 4 | ![Diagram](image4) | Mid-centrum thickness |
| Slenderness Ratio   | Dorsal length/ Posterior width  
Length of line 1/ Length of line 4 | ![Diagram](image5) | Vertebral slenderness |
Table 5.2: Vertebrae with significant values from ratio tests

<table>
<thead>
<tr>
<th>Statistical test</th>
<th>Comparison 1 Non-curved females vs Non-curved males</th>
<th>Comparison 2 Curved females vs Curved males</th>
<th>Comparison 3 Curved males vs Non-curved males</th>
<th>Comparison 4 Curved females vs Non-curved females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t-test</td>
<td>Levene</td>
<td>t-test</td>
<td>Levene</td>
</tr>
<tr>
<td>Length Ratio</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
</tr>
<tr>
<td>Width Ratio</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
</tr>
<tr>
<td>Height Ratio</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
</tr>
<tr>
<td>Thickness Ratio</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
</tr>
<tr>
<td>Slenderness Ratio*</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
<td>NS for all vertebrae</td>
</tr>
</tbody>
</table>

Each vertebra was examined using both a t-test and Levene test, for each comparison. A sequential Bonferroni correction was used to adjust significance levels (where \( \alpha = 0.05 \) and \( n = 40 \) ([4 comparisons x 2 tests) for each ratio x five ratios]). * Because vertebra 8 has a reduced anterior width, slenderness was tested using posterior widths.
CHAPTER 6: DISPROPORTIONATE BODY LENGTHS CORRELATE WITH IDIOPATHIC-TYPE CURVATURE IN THE CURVEBACK GUPPY*

*A version of this chapter is an accepted manuscript and will appear as: as KF Gorman and F Breden 2009. Spine.
6.1 Abstract

**Study Design.** A comparative allometric study of body lengths in an animal model for human idiopathic-type scoliosis.

**Objective.** To compare body length variation among adult curved and non-curved *curveback* female guppies.

**Summary of Background Data.** Tallness and/or abnormal anthropometric parameters have been correlated to idiopathic-type scoliosis (IS) in numerous studies. Heritable curvature in *curveback* has demonstrated morphological and developmental similarities to human IS. Because control of body length in the guppy is heritable, we investigated whether length might also be correlated to curvature in the *curveback* population.

**Methods:** Component body lengths were measured from digital photographs for 321 (246 curved and 75 non-curved) females. Sources of experimental variation were omitted by only measuring two-dimensional curves in mature females all from the same pedigree, and raised under controlled conditions of diet and environment. Body length was divided into two component parts (precaudal and caudal). Body lengths were tested statistically for correlation to curvature and curve magnitude.

**Results:** Although absolute length does not correlate to curvature, this survey of length in the *curveback* model reveals two important similarities to anthropometric studies of IS: that there are disproportionate body lengths among females with curvature, and the suggestion of an underlying growth abnormality among curved individuals.
Conclusions: In order to better characterize the relationship between growth, length disproportion and curvature in the guppy, further studies are warranted. However, this inquiry further supports the usefulness of curveback as a model for understanding the basic biology of idiopathic-type scoliosis and encourages study of growth-related factors.

6.2 Introduction

In both humans and guppies, idiopathic-type spinal curvature is a multifactorial deformity that exhibits extensive phenotypic variation. This variation is likely a consequence of interactions among genetic, physiological, and environmental factors. Success at identifying the aetiology of human idiopathic-type scoliosis (IS) has been limited due to trait complexity and the lack of animal models with a non-induced phenotype similar to humans [1]. Preliminary studies in otherwise healthy guppies have revealed that heritable curvature in curveback has remarkable morphological and developmental similarities to human IS (e.g., no vertebral fusion or breaks, distortion of apical vertebrae, curve onset after birth, variable rates of progression and prognoses for curve magnitude, a female bias for severe curves) [2,3]. As with humans, control of body length in the guppy is heritable [4-6]. Here we explore length variation in the curveback population, because numerous studies have associated IS with tallness and/or abnormal length proportions along the cranio-caudal axis [7-24].

We surveyed adult curveback females for increased length, because mature females with IS have demonstrated increased cranio-caudal length [7]. We show that among curved fish, the caudal portion of the body is
disproportionately longer relative to the pre-caudal body, and this disproportion is positively correlated to curve magnitude. As with anthropometric studies, these results suggest that a growth abnormality may be associated with idiopathic-type spinal curvature.

### 6.3 Materials and Methods

#### Study population and data collection

Guppies are live-bearing teleost fish; offspring are born with a fully ossified skeleton after ~ 3 weeks of gestation. Curvature begins after birth and is generally stable by sexual maturity (approximately 1 month past birth) [3]. In cases of severe curvature, curves will progress into early adulthood (up to 2 months after birth). Breeding pairs were maintained in 4L plastic aquaria, and offspring were separated into individual 600 ml plastic containers after birth. All fish are kept under standardized conditions (i.e., flakes fed every afternoon, supplemented with brine shrimp nauplii; 25-26° C; pH 7-9; RO water reconstituted to 1600-1800 ppm salinity with aquarium salts; 14/10 hour light cycle). Adult females were euthanized at a minimum of 3 months past birth. Males were not measured because adult coloration obscures the spine. Euthanized females were photographed on a light table with a digital camera (Toshiba PDR-3310, NYC, USA) under 3X magnification.

We measured 246 adult females with curves of varying magnitude, and 75 normal adult females, all from the same pedigree. Curvature is manifest as a sagittal anterior lordosis and posterior kyphosis. A small number of fish exhibit
curvature in the frontal or horizontal planes, and in this study these were omitted. The distribution of curve magnitude is shown in figure 6.1, with curve magnitude identified by qualitative scores defined in Gorman, et al., 2007, in which 0 denotes no curvature and 1-4 is a scale of increasing magnitude.

To assess whether there are length differences between curved and normal curveback females, we divided the body into two components along the cranial-caudal axis: the precaudal body and the caudal body (figure 6.2). Broadly speaking, these two component lengths are comparable to how the trunk and the legs comprise the cranial-caudal lengths measured in anthropometric studies of IS (e.g., chepalo-caudal proportions in Nicolopoulos, et al, 1985). In the guppy, the pre-caudal body contains rib-associated vertebrae. In curveback, curvature is manifest in the caudal body, where vertebrae are not associated with ribs. The precaudal length plus the caudal length comprise the ‘standard length’, which is a standard measurement in fish biology.

**Measurements**

All fish were positioned on their side above a ruler so that there was no variation in orientation. All photographs were taken from above so that the camera looked down on the profile of the fish. To ensure consistency, photographs were taken by the same person throughout the experiment. Photographs were scaled and measured using Image-J (NIH Image) [25]. Fish with coronal curvature or axial rotation are not included in the dataset because in photographs, the sagittal profile of the spine would be distorted and measurements would not accurately reflect length.
Body portions were measured as the length of a line drawn between landmark points. Because curvature is located in the caudal portion of the body, we defined the ‘manifest length’ as the length of a line drawn directly between landmark points, and the ‘true length’ as the length of a line traced along the spine within the caudal body. These length measures are similar in concept to the uncorrected and corrected lengths used in anthropometric studies, the difference being that we do not correct for curvature with the formula of Bjure (1968) [26]. The true caudal length (line 1 in figure 6.3) is the length of a line drawn along the ventral edge of the spine between the top of the cloacal vent to the bottom tip of the caudal peduncle (point A to point B in figure 6.3). The manifest caudal length (line 2) is the length of a line drawn directly from points A and B. In a non-curved fish, the manifest length is equal to the true length (i.e., the length of line 1 = the length of line 2). The precaudal body length is the length of a line drawn from point A to the anterior-most tip of the nose (point C, line 3 in figure 3). The manifest standard length (line 4 in figure 6.3) is defined as the length of a line drawn from the tip of the nose to the caudal peduncle (point C to point B in figure 3), and is analogous to the uncorrected standing height in human studies. The lengths of line 1 plus line 3 comprise the true standard length of the fish. In a curved fish the length of line 4 is less than the length of line 1 plus line 3.

The Shapiro-Wilks test was used to test the distributions of length measurements among normal and curved females for normality. Given that most distributions were significantly non-normal (P < 0.05), mean values were
compared via Wilcoxon tests with alpha set at 0.05 ($\chi^2$ approximation, 1 d.f.). To explore the relationship between length measures, simple linear regression and analysis of covariance (ANCOVA) of log-transformed values were used. As defined by Huxley (1932), the log values of the lengths of two body components plotted against each other give a slope of 1 if there is no allometric growth [27]. Statistical analysis was conducted using JMP software for MacOSX, Version 6.0, SAS Institute, INC., Cary, NC, USA.

6.4 Results

We first tested whether there are differences between curved and non-curved fish for each length measurement (table 6.1 and figure 6.3). Wilcoxon pair-wise comparisons show that there is no statistical difference between normal and curved females for mean values of: manifest standard length (line 4); precaudal body (line 3); and manifest caudal length (line 2). However, there is a significant difference between normal and curved females for the true caudal length (line 1) and true standard length (line 1 + line 3).

To test whether the length of the caudal spine is related to curve severity, we tested for allometry between the true and manifest caudal lengths by linear regression on log-transformed values. In curved fish, if there is an allometric relationship between the lengths of lines 1 and 2, then a slope greater than one would show that true caudal length increased faster than manifest caudal length, i.e. that curve severity increased with the length of the caudal spine. Among normal fish, the slope of this line is expected to be one. So, to test for significant differences, we compared the slope for all curved fish to the slope for all normal
fish with an ANCOVA. We found that among curved fish, the true caudal length (line 1) increases in proportion with the manifest caudal length (line 2) indicating that a longer caudal spine is not correlated to curve progression [slope for non-curved=0.99 ($R^2=1.0$); slope for curved=0.93 ($R^2=0.82$); ANCOVA $P=0.23$].

To investigate the relationship between the length of the caudal spine and the length of the precaudal body, we tested for allometry between the lengths of line 1 and line 3. We found that curved fish have significantly longer caudal spines coupled with shorter precaudal bodies, and that this disproportion is greater among larger females (ANCOVA; $p=0.0021$) (figure 6.4). Our preliminary analysis identified 5 individuals who exhibited extremely small precaudal bodies coupled with long caudal spines. These outliers were omitted from our analysis of the regression shown in figure 6.4, but are considered in figure 6.5 and in the discussion.

The regression shown in figure 6.4 shows that females with higher curve magnitudes have longer relative caudal spines. Therefore, the relationship between the relative caudal length and curve magnitude was further investigated by comparing mean values for the caudal portion (the length of line 1/the length of line 3) for each qualitative category of curve magnitude to the mean caudal portion for normal females. Figure 6.5 demonstrates that among curved females, as curve magnitude increases, the difference between the mean caudal portion for curved fish compared to normal also increases (figure 6.5).
6.5 Discussion

The present study investigated whether body length might correlate with curvature in the *curveback* guppy model. Our analysis shows that there is no difference between mature normal and curved females for mean values of manifest (i.e., uncorrected) standard and caudal lengths, or precaudal body length. However, there is a significant difference between normal and curved females for true caudal length, although it does not correlate with curve magnitude. Furthermore, there is an allometric relationship between the length of the caudal spine and the length of the precaudal body such that adult curved females demonstrate a disproportionately longer caudal spine relative to the precaudal body length, and this is more pronounced among larger fish and those with greater curve magnitudes.

That a longer caudal spine correlates to curvature generally, but not curve magnitude, suggests that it is not a risk factor for severe curvature. However, disproportionate lengths could be indicative of an underlying growth abnormality that is a risk factor associated with severe idiopathic-type curvature. This is suggested by: 1) disproportion increases with curve magnitude, 2) disproportion is more pronounced among larger fish, and 3) the apparent growth abnormality present in the outliers in figure 6.5. Our analysis of relative caudal length shows five fish of high curve magnitude that have extreme disproportion (outliers in figure 5.5, curve score 4), such that the true caudal length is 75-100% of the value of the precaudal length (compared to ~57% in non-curved). Upon inspection, these females have abnormally small precaudal bodies that suggest
an exaggerated growth abnormality (figure 6.6). If there is a genetic basis for uncoordinated growth that is linked to curvature, then within the inbred *curveback* pedigree we expect to observe extreme cases of disproportion as we select for higher magnitudes of curvature. Although this appears to be the case, further analysis of inheritance for body length in the *curveback* pedigree is necessary.

**Body lengths, growth, and human idiopathic-type curvature**

Many human studies have correlated disproportionate lengths along the cranio-caudal axis with IS [7, 13, 14, 16, 21-23]. However, inconsistencies between studies in sample sizes and methodology (e.g. at what age a cohort is measured and whether to calculate pelvic height as a component of stature outside of sitting and standing heights) have led to different conclusions regarding which specific body component (trunk or leg length) is relevant, and exactly how the disproportion relates to growth, age and maturity (e.g., whether pubertal status is considered as a maturity marker in addition to age). Further potential for inconsistencies between IS studies is introduced by the fact that methods of correction for loss of height due to curvature use two-dimensional measurements of a three-dimensional deformity [26, 28]. This means that as the magnitude of the deformity increases, the power to detect an accurate relationship with height/length decreases.

With human IS, the length of the spine has been positively correlated with curve magnitude. However, the methodology behind this correlation needs verification because the commonly used Bjure formula introduces a spurious positive correlation between corrected height and curve
magnitude (e.g. Cheng, et al., 2003) due to the fact that Cobb’s angle is used to correct height measurements (Logy=0.011x-0.177; where y is the loss of trunk height caused by curvature and x is the Cobb angle of the primary curve) [26]. Thus both the dependent and independent variables include curve magnitude.

Despite their inconsistencies, all anthropometric studies address the likelihood that disproportionate lengths in girls with IS are a consequence of abnormal growth patterns. Furthermore, studies that measure females during growth suggest that in cases of IS, abnormal prepubescent stature proportions (trunk or leg lengths) might be related to androgenic affects [7,16,29]. Endocrine factors such as growth hormone activity [29-33], estradiol levels [34,35], or testosterone levels [31,35] have been considered as possible risk factors for curve progression in IS. Given that the androgenic and somatotrophic axes are the major hormonal systems regulating postnatal linear growth and have principal roles in regulating skeletal growth and bone mass [36], it is possible that androgenic and/or somatotrophic pathway interactions are involved in disproportionate cranial-caudal lengths associated with idiopathic-type curvature. For example, associations have been found between allelic variants of the oestrogen receptor gene (Pvull), growth hormone receptor gene polymorphisms, and adult female height [37-39]. Without an animal model however, hypotheses regarding the effects of these hormones have been difficult to test.

6.6 Conclusions

As a model, the curveback guppy can be used to control for sources of experimental inconsistency, and thus give insight into the relationship between
variables such as body lengths, curvature, and curve magnitude. In the present study, possible sources of experimental variation were eliminated by limiting our sample to mature females with two-dimensional curvature, all from the same pedigree, and grown under controlled conditions of diet and environment. However, by restricting our dataset to mature females, we could not detect important growth-related variation that might be related to body length disproportion and/or curvature. For example, in their comprehensive anthropometric study, Cheng, et al. (2003) demonstrated that females with IS were shorter and leaner than maturity-matched controls at their prepubertal growth spurt, but then had significantly greater corrected heights, sitting heights, and segmental lengths (arm span and leg length) after puberty. We therefore suggest further study into the relationship between body lengths and curvature throughout curveback growth. Furthermore, using curveback families that are inbred for either high or low curve penetrance and expression, it is feasible to experimentally manipulate androgens and/or growth hormone during growth to determine the extent to which these influence length proportions.

Our survey of body lengths in 321 curveback females reveals two important similarities to anthropometric studies of IS: that there are disproportionate cranial-caudal lengths among females with curvature, and the suggestion of an underlying growth abnormality among curved individuals. These similarities are interesting considering that androgenic and somatotrophic axes are the major hormonal systems regulating postnatal linear growth in all vertebrates, and that recent inquiries reveal extensive conservation of endocrine
regulation between humans and teleosts [40-41]. Therefore, this inquiry further supports the usefulness of curveback as a model for the basic biology of idiopathic-type scoliosis and encourages study of growth-related factors.

6.7 Acknowledgements

We acknowledge CC Tsai at Simon Fraser University. This study received support from the Scoliosis Research Society, Natural Sciences and Engineering Research Council of Canada, and the US National Institute of Health (#1R21AR053730-01A2). This research was approved by the SFU University Animal Care Committee, project #763B.

6.8 Literature cited


39. Audi L, Esteban C, Carrascosa A, et al., Exon 3-deleted/full length growth hormone receptor polymorphism genotype frequencies in Spanish short small-for-gestational age (SGA) children and adolescents (n=247) and in adult control population (n=289) showed increased fl/fl in short SGA. *J Clin Endocrinol Metab.* 2006; 91: 5038-5043.


Figure 6.1 Distribution of Curve Magnitude within Study Population

Normal fish have a qualitative score of 0; curved fish were scored as 1-4 representing increased curve magnitude.
Bodies of *curveback* adult females were divided into precaudal and caudal portions: the precaudal portion extends from the tip of the nose to the top of the cloacal vent, and the caudal body portion extends from the top of the cloacal vent to the tip of the caudal peduncle. Scale shown in cm.
Figure 6.3 Component Measures

Line 1: *true caudal length*- length of a line drawn along ventral edge of the spine from the top of the cloacal vent (point A) to the tip of the caudal peduncle (point B). **Line 2**: *manifest caudal length*- length of a line drawn directly from point A to point B. In a curved fish the length of line 2 is less than the length of line 1. **Line 3**: *precaudal body length*- length of a line drawn from the top of the cloacal vent (point A) to the tip of the nose (point C). **Line 4**: *manifest standard length*- the length from point C to point B.
There is a significant difference between curved (solid line) vs. non-curved (dotted line) females for relationship of true caudal length (log of line 1) and precaudal length (log of line 3): [slope for non-curved=0.75 (R²=0.87); slope for curved= 0.9 (R²=0.79); ANCOVA; p=0.0021]. Curved individuals are denoted as circles: large solid circles= higher magnitudes (scores 3 & 4), open circles= moderate magnitudes (score 2), small dots= low magnitudes (score 1); normal individuals denoted by a black 'x'.
Figure 6.5 Caudal portion increases with curve magnitude

Wilcoxon pair-wise comparisons of mean caudal portion (line 1/line 3). Each panel shows the caudal portion values for all individuals within a category for curve magnitude (qualitative scores shown in figure 1), compared to caudal portion values for non-curved fish (mean values connected by line). As curve magnitude increases, the difference between caudal portion and normal increases. Bars of one standard deviation are shown. Note outliers for curve magnitude 4 individuals.
A: One of the outliers in figure 5. All outliers have a similar morphology.

B: Age-matched female Showing typical curveback phenotype. Scale shown in mm.
Table 6.1 Comparison of length measurements in curved versus non-curved fish.

<table>
<thead>
<tr>
<th>Length measure (mm)</th>
<th>Mean value (SE)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal</strong></td>
<td><strong>Curved</strong></td>
<td></td>
</tr>
<tr>
<td>Manifest Standard length (line 4)</td>
<td>23.2(0.39)</td>
<td>NS (p=0.82)</td>
</tr>
<tr>
<td></td>
<td>23.2(0.20)</td>
<td></td>
</tr>
<tr>
<td>True Standard length (lines 1 + 3)</td>
<td>23.2(0.37)</td>
<td>p=0.05</td>
</tr>
<tr>
<td></td>
<td>24.1(0.20)</td>
<td></td>
</tr>
<tr>
<td>Precaudal length (line 3)</td>
<td>14.9(0.28)</td>
<td>NS (p=0.48)</td>
</tr>
<tr>
<td></td>
<td>15.1(0.13)</td>
<td></td>
</tr>
<tr>
<td>Manifest caudal length (line 2)</td>
<td>8.31(0.13)</td>
<td>NS (p=0.39)</td>
</tr>
<tr>
<td></td>
<td>8.43(0.07)</td>
<td></td>
</tr>
<tr>
<td>True caudal length (line 1)</td>
<td>8.35(0.13)</td>
<td>p&lt;.0001*</td>
</tr>
<tr>
<td></td>
<td>9.01(0.07)</td>
<td></td>
</tr>
</tbody>
</table>

Significance tested by Wilks using $\chi^2$ approximations with 1DF.
CHAPTER 7: MAPPING LOCI ASSOCIATED WITH CURVEBACK*

* Because a patent application will be submitted based on the results in this chapter, the chromosomal position of the QTL identified is not given.
7.1 Abstract

Neither the genetic basis nor the biological processes involved in the aetiology of idiopathic-type scoliosis are known. As a consequence, the human idiopathic scoliosis syndrome has remained an enigma since its first documentation by Hippocrates. The fact that the pathogenesis coincides with growth, that there is a large amount of phenotypic variation, and the lack of a genetic and developmental animal model have all contributed to difficulty in understanding the etiopathogenesis. The curveback guppy is the only animal that demonstrates heritable idiopathic-type scoliosis with developmental similarities to the human syndrome. Genes that are associated with idiopathic-type scoliosis in curveback will identify biological pathways involved in this complex deformity in the guppy, and thus suggest candidate genes and possible biological pathways involved in the human deformity. Here we describe the mapping of major chromosomal regions associated with curvature in the curveback guppy. Using the high-density genetic linkage map for the guppy, we screened 84 backcross individuals with 367 genetic markers to identify two loci that are significantly linked to curvature.

7.2 Introduction

Human idiopathic-type scoliosis (IS) is a heritable disorder that affects an average of 3%-4% of the global paediatric population. Although a genetic basis is acknowledged (reviewed in Wise, et al., 2008; see chapter 1), the aetiology for this deformity has been unknown since its first description by Hippocrates (see Postacchini, 1995). The lack of progress in understanding causative factors is
likely due to trait complexity and the lack of a genetic and developmental animal model.

The *curveback* guppy is the first animal model with heritable morphological and developmental similarities to IS, and thus provides a means to investigate the biological processes that cause idiopathic-type scoliosis (Gorman, *et al.*, 2007; Gorman and Breden, 2009a; 2009b). Identification of genes that cause curvature in the *curveback* model has the potential to elucidate candidate biological pathways involved in idiopathic-type curvature in the guppy, which will provide strong candidates for the human deformity. Therefore, genes discovered in *curveback* could lead to effective screening to facilitate early curve detection and therapies in humans. There is currently no method for early screening in humans. The goal of this study was to identify major chromosomal region(s) that are correlated curvature in *curveback*.

### 7.3 Materials and Methods

**Mapping crosses and phenotypic evaluation**

An extremely curved *curveback* male was crossed to a normal and unrelated female from a lineage having no prior incidence of spinal curvature. The maternal lineage is a mixture of laboratory fish derived from Central Cumaná, Venezuela and a pet store fancy guppy stock, and has been inbred (via sibmates) for 7 generations as part of another experiment. According to the qualitative scale for curve magnitude (Gorman *et al.*, 2007), the *curveback* male had a magnitude of 4 (the highest curve magnitude), and was affected in the coronal and sagittal
planes (the maternal phenotype had a score of 0). All fish were kept under standardized conditions (i.e., flakes fed every afternoon, supplemented with brine shrimp nauplii; 25-26°C; pH 7-9; RO water reconstituted to 1600-1800 ppm salinity with aquarium salts; 14/10 hour light cycle). Breeding pairs were maintained in 4L plastic aquaria, and offspring were separated into individual 600 ml plastic containers after birth. Fish were scored for curvature throughout growth according to the qualitative methods described in Gorman, et al. 2007 (Chapter 3).

From the parental cross 30 F1 offspring were produced, all of which showed no curvature. Two normal F1 daughters were subsequently backcrossed to their curved father to obtain a total of 129 backcross (BC) mapping progeny, of which 48% have curves of variable magnitude. In addition, two normal F1 progeny were crossed to create 286 F2s, of which 22% showed curves of varying magnitude. The distribution of curve magnitude among BC and F2 offspring is given in table 7.1. The two parentals, all of the F1s, the BC, and F2 offspring have been photographed on a light table with a digital camera (Toshiba PDR-3310, NYC, USA) under 3X magnification, and frozen in buffered (EDTA) ethanol. Adult BC and F2 mapping offspring were euthanized at a minimum of 3 months past birth (sexual maturity is at about 1 month past birth).

**Genotyping BC offspring**

To map chromosomal regions that are associated with idiopathic-type curvature in *curveback*, two 96-well plates comprised of DNA from the 2 parentals; 9 F1 males and females (including those used in production of BC and
F2 offspring), and 53 normal and 31 curved BC offspring, were genotyped by Sequenom, Inc., using guppy-specific SNP (single nucleotide polymorphism) markers (Tripathi, et al., 2009). DNA was extracted from tissue using the DNeasy Blood and Tissue kit and protocol (Qiagen), and diluted as per specifications from Sequenom. The backcross offspring are from two families: 54 from BC1 (20 normal females and 13 normal males; 13 curved females and 8 curved males) and 30 from BC2 (18 normal females and 2 normal males; 7 curved females and 3 curved males).

The first plate was genotyped in 2007 with 189 EST-based (expressed sequence tag) SNP markers (the total number of EST markers available). These SNP markers are optimized for high-throughput genotyping from Sequenom Inc. using mass spectrometry. Because the plate was processed before the guppy linkage map (Tripathi, et al., 2009) was available, we had no knowledge of where in the guppy genome these markers were located. In 2008, a second plate composed of the same individuals, was genotyped with 178 additional genomic SNP markers. In collaboration with Dr. Detlef Weigel and Dr. Christine Dreyer at the Max Plank Institute in Tübingen, we were able to use the guppy linkage map (Tripathi, et al., 2009) prior to publication to describe the location of the EST-based markers from our first plate, and then choose additional markers for the second plate. Markers from the second plate were chosen with the goal of creating a map with 10 cM resolution.
Statistical analysis

The genotype data from both plates were combined for analysis. Estimates for genotype-phenotype correlations were made using the interval mapping functions from the web-based software, QTL Express, available at http://qtl.cap.ed.ac.uk/ (Seaton, et al., 2002). The program uses a multiple linear regression analysis method that was first developed for inbred lines (Haley and Knott, 1992) but then extended for outbred lines (Haley, et al., 1994). Although outbred, it is assumed that the two mapping lines are fixed for alternative alleles at QTL. This analysis is suitable for the curveback mapping data because although inbred for several generations, the parental lines are not homozygous at all marker loci.

The analysis first determines transmission probabilities (probabilities of inheriting 1 or 2 alleles from the curveback parent) for BC offspring at every cM, conditional on flanking informative marker genotypes and marker position. Then the phenotypes are regressed onto these probabilities. For curveback, we tested sagittal curvature and coronal deviation as two separate traits, in case these components of the overall phenotype were influenced by different loci. A single locus or two QTLs are fitted to the linear model. We first conducted analyses under the single locus model, and then under the two QTL model. For each putative QTL, an additive effect was estimated. The additive effect (i.e. substitution effect) for each allele is defined by the difference between the recurrent QTL genotype and the heterozygous genotype. For example, in the curveback backcross, the allele substitution effect would be the difference
between CC and NC (CC-NC, where C denotes an allele from the *curveback* parent and N denotes an allele from the normal parent). A positive value for the allele substitution effect means that the C allele increases the phenotype (magnitude of curvature). Sex was included as a fixed effect, and we also tested for an interaction between putative QTLs and sex. An *F*-ratio was calculated for every cM and the most probable position of a QTL is considered to be at the location on a chromosome with the highest *F*-value. To determine significance thresholds, one thousand permutations per chromosome (linkage group) were carried out for each trait, and a mean was computed to provide the *F*-statistics under the null hypothesis that no QTL was segregating on that chromosome (Churchill and Doerge, 1994).

The proportion of variance explained by putative QTL was estimated using a one-way ANOVA with the BC genotypes for each marker/locus (see results) as one factor. These effects were estimated as the ratio of the Sum of Squares/Sum of Squares Total. In order to detect loci that might be masked by the effects of a major QTL, further genomic analysis was conducted using the genotypes for Markers 1 as a fixed effect (see results). In order to detect loci that might be masked by Marker 1 and Marker 2 genotypes, a third genomic analysis was conducted using the genotypes for markers 1 and 2 as fixed effects (see results for details). Also, markers at putative QTL were tested for an interaction with sex using a two-way ANOVA. This is in addition to the analysis carried-out by QTL Express (test for an interaction of linked marker genotypes and sex). A two-way ANOVA was used to test for a significant interaction between the genotypes of
Marker 1 and Marker 2 (see results) that might affect the phenotypic variance. All ANOVA functions were conducted using JMP statistical software for MacOSX, Version 7.0, SAS Institute, INC., Cary, NC, USA.

7.4 Results

The guppy genome consists of 23 haploid chromosomes (Winge, 1922), with a genetic map distance that is estimated at 899 cM, and no linkage group (LG) longer than 58 cM (Tripathi, et al., 2009). A total of 367 guppy-specific SNP markers were tested on the 2 parental, 9 F1, and 84 BC offspring. From these, 148 markers (40%) were polymorphic in either one or both of the mapping crosses, 141 of which could be assigned to the 23 guppy linkage groups (69 markers are from ESTs, 72 are genomic, and 7 are unassigned EST-based markers). With the exception of LG 3, which has one marker, the number of markers per LG ranges from 3-12 and the size of intervals between markers ranges from 0.1-19.57 cM (table 7.2).

Analysis of the genotypes (from plate 1 and plate 2 combined) with QTL Express identified one major effect locus (called Locus1 on LGa) that has a significant association with sagittal curvature. Locus 1 is located at the end of an interval and so is synonymous with Marker 1 on LGa. It is significant at both the chromosome-wide 5% and 1% thresholds, and explains an estimated 37% of the total phenotypic variance (figure 7.1). Linkage group 3 was omitted from the interval analysis because it only contained one marker, which is insufficient for interval mapping. A one-way ANOVA that tested for an association between the
marker on LG3 and curve magnitude showed no linkage (p= 0.26). Coronal curvature was not significantly associated with any marker.

To test for loci that are associated with curvature but masked by the effects of Marker 1, we did a second genomic analysis in QTL Express with Marker 1 modelled as a fixed effect (in addition to sex). From this analysis, a second locus became evident. Locus 2, located on LGb, is also located at the end of an interval and is synonymous with Marker 2. This locus is significant at the 0.5% level, but not at 0.01%, and explains an estimated 7.6% of the total phenotypic variance (figure 7.2). Interval analysis showed no marker genotype that had a significant interaction with sex, and this was confirmed for Markers 1 and 2 by a two-way ANOVA (Marker 1*sex: p=0.89; Marker2*sex: p=0.27). The results of the QTL analysis are summarized in table 7.3.

A two-way analysis of variance (ANOVA) showed a significant interaction between Marker 1 and Marker 2 for curve magnitude (p=0.013). This interaction identifies Marker 2 as a modifier for curve magnitude in the presence of Marker 1. When an individual is curved (i.e. is homozygous for the ‘T’ allele at the Marker 1 SNP), and also homozygous for the ‘C’ allele at Marker 2, then the average magnitude of curvature is slightly increased. The average magnitude is further increased for individuals who are homozygous for the ‘G’ allele, and the greatest for individuals who have a ‘CG’ genotype for the Marker 2 SNP. The genotype of the curved paternal father was ‘TT’ for Marker one, and ‘CG’ for Marker 2; the normal maternal genotype (Pfemale, not F1 female) was ‘AA’ for Marker 1 and
‘CC’ for Marker 2. If an individual is non-curved (i.e. is ‘TA’ for Marker 1), then the genotypes for Marker 2 have no effect (figure 7.3).

7.5 Discussion

Based on phenotypic complexity observed in the *curveback* pedigree (e.g. variable age of onset, variable rates of curve progression, and variable final curve magnitude), we presumed that idiopathic-type curvature in *curveback* is under the control of multiple loci. That curved individuals in the BC and F2 mapping crosses show variation for adult curve magnitude further supported this idea. As an initial step in mapping candidate genes for idiopathic-type spinal curvature in the guppy, we used interval mapping (Lander and Botstein, 1989) to identify 2 chromosomes that are significantly linked to the *curveback* phenotype. To detect and localize QTLs in a single mapping effort, large numbers of individuals and markers would be necessary. Because the mapping efforts described in this study do not involve a large number of individuals (i.e. 84 BC offspring), there will likely be multiple phases involved in fine mapping for curvature. The fact that QTL mapping is an iterative procedure that typically requires multiple steps is characteristic of QTL studies (Liu, 1998; MacKay, *et al.*, 2009).

Combined, the individual effects of Locus 1 and Locus 2 explain an estimated 44.6% of the total phenotypic variation. However, a two-way ANOVA shows a significant interaction between the two loci that may explain a greater proportion of the phenotypic variance. Further unexplained phenotypic variability may derive from: 1) undetected modifier loci, 2) undetected genetic interactions, 3) the environment, or 4) epigenetics. A limitation with backcross populations is
that they contain only a subset of the possible genotypes at a locus (e.g. in a cross between parental genotypes CC and NN the F1 are CN, and the backcross genotypes can only be CN or CC). As a consequence, a BC mapping design does not provide information about allelic interactions (i.e. dominance).

We chose to first identify major loci associated with curveback using a backcross design because we expected to obtain more curved offspring, which would increase our power to detect major effect loci. Based on the 30 normal F1 from the parental cross in this experiment, and evidence from the curveback pedigree (Chapter 1: 6 F1 of the original pedigree cross are normal), we presume that the genetic basis of curveback is recessive. Because there is a greater probability of obtaining homozygous progeny from a BC compared to an F2 cross, we expected a greater number of curved individuals in our BC progeny. This was in fact the case (see table 7.1).

**The next step for mapping QTL**

Interval mapping analysis identified two positions that are linked to curvature on separate linkage groups. The next step in mapping will be to define the loci located on LGa and LGb. To accomplish this we will need additional markers surrounding Marker 1 and Marker 2. Markers that diminish in their linkage to curvature will define locus boundaries. This will be evaluated by the number of recombinant genotypes for markers in close proximity to the loci. There are 22 SNP markers untested on LGa and 19 untested SNP markers on LGb. The primers for the context sequences in which each SNP is contained is available from Tripathi et al. (2009), and so we can sequence the maternal and
paternal genomes per marker and select suitable SNPs for our mapping population. Genotypes can be assayed either by PCR-RFLP mapping (Ota, et al., 2007) or TaqMan SNP detection (ABI).

In addition to more markers tested on BC offspring, we will need additional mapping individuals to increase the precision of the defined loci. Therefore, we will genotype the remaining BCs (N=45) and the F2's described in table 1.1 (N=286). This will allow us to confirm the loci identified in this study with BCs, increase the probability of obtaining recombinants for markers close to the loci of interest, as well as allow us to detect genetic interactions. We hope that these efforts will refine the loci so that we identify as small of a number of candidate genes as possible. In an organism with a well-annotated genome, a query of candidate genes in the QTL can be made to identify likely causal genes. However, the guppy does not have a sequenced genome.

Although a fully annotated guppy genome is not available, synteny to the medaka genome makes identification of candidate genes in the guppy possible. The guppy and medaka are very closely related species. A supertree phylogeny by Mank et al. (2005) places beloniformes (medaka) and cyprinodontiformes (guppy) as sister orders in the superorder Acanthopterygii. Tripathi, et al (2009) has demonstrated genome-wide synteny between the guppy and medaka with the SNP markers that we used for our curveback mapping. Of 332 guppy-specific markers linked to coding genes with significant sequence similarity in the medaka, 276 could be assigned to guppy LGs, with between 2 and 16 markers belonging to a single medaka chromosome. In this study, Marker 1 happens to
be located in the middle of a syntenic block of EST-based guppy markers, and Marker 2 is located within 1cM of a syntenic block. Hence, once we refine the loci identified, it is feasible that we will be able to use the medaka genome to identify candidate genes.

**How heritable idiopathic-type scoliosis in the guppy can be applied to IS**

Mapping genes for spinal curvature in *curveback* will determine the molecular pathway(s) leading to spinal curvature in the guppy, and these findings will provide strong candidate genes for IS in humans. Identification of genes associated with IS could allow for effective screening to facilitate early curve detection in humans. Presently there is no method of screening for curvature other than the forward bending test invented by Adams in 1865 (see Fairbank, 2004 for historical account). In 2005 the US Preventive Services Task Force recommended against routine IS screening, because the forward bending test has been found to be ineffective at curve detection and may even lead to moderate harm, through unnecessary brace wear and unnecessary referral for specialty care (www.ahrq.gov/clinic/3rduspstf/scoliosis/scoliors.htm#background). Prediction of curve predisposition would allow for targeted monitoring of curves and would eliminate unnecessary exposure to X-rays by eliminating false positives.

**7.5 Conclusions**

This chapter describes the initial steps for mapping genes that cause idiopathic-type spinal curvature in the *curveback* guppy. The initial step in the
identification of QTL is the analysis of data to find major linked loci. For this purpose, interval mapping is adequate (Liu, 1998). By interval mapping, we have identified 2 guppy-specific genomic SNP markers that are putative QTL for idiopathic-type curvature in the curveback guppy. The independent effects of these loci account for an estimated total of 44.6% of the phenotypic variance. Moreover, there is a significant interaction between these loci that affects curve magnitude. To generate the data for this analysis, we used 84 BC offspring. In order to fine map the chromosomal regions identified, we will use additional markers and individuals from the F2 cross. Analysis of F2 data will allow us to explore genetic interactions in greater detail.

7.6 Acknowledgements
Support from Natural Sciences and Engineering Research Council of Canada; and Award Number R21AR053730 from the National Institute Of Arthritis And Musculoskeletal And Skin Diseases. I would like to thank Dr. Felix Breden, Dr. Julian Christians, Roozbeh Amahadi, Corey Watson, and Jennifer Parent for their significant contributions to this chapter.

7.7 Literature Cited


Gorman and Breden 2009b. Disproportionate body lengths correlate with idiopathic-type curvature in the curveback guppy, Spine (accepted manuscript, April 2009).


Figure 7.1: Marker 1 on linkage group ‘a’

Marker 1 is significantly linked to curvature at the 5% (solid, red line) and 1% (dashed, blue line) significance levels, and explains an estimated 37% of the total phenotypic variance. Figure generated from QTL Express. Variance ratio denotes the F-statistic (Y-axis), chromosomal positions for LGa on the X-axis.
Figure 7.2: Marker 2 on linkage group 'b' 

Marker 2 is significantly linked to curvature at the 5% (solid, red line) significance level (dashed, blue line shows 1% significance threshold), explains an estimated 7.6% of the total phenotypic variance. Figure generated from QTL Express. Variance ratio denotes the F-statistic (Y-axis), chromosomal positions for LGb on the X-axis.
The interaction between Marker 1 and Marker 2 is such that Marker 2 is a modifier for curve magnitude in the presence of Marker 1. When an individual is homozygous for the 'T' allele at the Marker 1 SNP (i.e. curved, red line), then the genotypes for Marker 2 modify curve magnitude. If an individual is 'TA' for Marker 1 (i.e. non-curved, blue line), then the genotypes for Marker 2 have no effect.
Table 7.1 Distribution of Phenotypes in mapping crosses

<table>
<thead>
<tr>
<th>Cross-type</th>
<th>Phenotypic Class (qualitative scores)</th>
<th>Total Offspring</th>
<th>Total Curved</th>
<th>Percent Curved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>0.5-1</td>
<td>1.5-2</td>
<td>2.5-3</td>
</tr>
<tr>
<td>Guppy BC</td>
<td>67</td>
<td>52%</td>
<td>32</td>
<td>25%</td>
</tr>
<tr>
<td>Guppy F2</td>
<td>224</td>
<td>78%</td>
<td>40</td>
<td>14%</td>
</tr>
</tbody>
</table>

The F1 from the parental cross (very curved *curveback* male x normal unrelated female) were used to create 2 backcrosses (data shown here is from the 2 crosses combined), and one F2 cross.


<table>
<thead>
<tr>
<th>Linkage Group</th>
<th>Number of markers</th>
<th>Smallest Interval (cM)</th>
<th>Largest Interval (cM)</th>
<th>Total Length*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>7.5</td>
<td>15.92</td>
<td>36.04</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.009</td>
<td>9.03</td>
<td>42.34</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>31.12</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>0.11</td>
<td>7.34</td>
<td>55.57</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4</td>
<td>9.34</td>
<td>32.41</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4.6</td>
<td>10.18</td>
<td>50.97</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>0.21</td>
<td>8.98</td>
<td>41.64</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>0.039</td>
<td>14.11</td>
<td>34.98</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>0.08</td>
<td>14.56</td>
<td>52.57</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>0.48</td>
<td>16.09</td>
<td>44.47</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>0.42</td>
<td>10.18</td>
<td>42.29</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>6.83</td>
<td>12.67</td>
<td>29.14</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>9.26</td>
<td>9.32</td>
<td>40.93</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>2.5</td>
<td>13.17</td>
<td>34.95</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>0.8</td>
<td>8.9</td>
<td>57.82</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>1.07</td>
<td>9.5</td>
<td>32.72</td>
</tr>
<tr>
<td>17</td>
<td>10</td>
<td>0.1</td>
<td>12.8</td>
<td>34.47</td>
</tr>
<tr>
<td>18</td>
<td>7</td>
<td>0.16</td>
<td>14.3</td>
<td>38.47</td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>0.7</td>
<td>19.57</td>
<td>31.21</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>0.41</td>
<td>9.7</td>
<td>35</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>5.5</td>
<td>13.8</td>
<td>39.01</td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>7.36</td>
<td>13.4</td>
<td>29.01</td>
</tr>
<tr>
<td>23</td>
<td>9</td>
<td>0.29</td>
<td>9.7</td>
<td>31.6</td>
</tr>
</tbody>
</table>

* According to Tripathi, *et al.*, 2009

With the exception of LG 3, which has one marker, the number of markers per LG ranges from 3-12 and the size of intervals between markers ranges from 0.1-19.57.
Table 7.3: Results from Interval mapping

<table>
<thead>
<tr>
<th>LG</th>
<th>Trait</th>
<th>F-statistic threshold</th>
<th>F-statistic</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p&lt;0.05</td>
<td>p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>sagittal curve</td>
<td>5.24</td>
<td>9.03</td>
<td>57.66</td>
</tr>
<tr>
<td>b</td>
<td>sagittal curve</td>
<td>2.71</td>
<td>4.22</td>
<td>3.19</td>
</tr>
</tbody>
</table>

Interval mapping has identified two markers that are statistically linked to curvature. Marker 1 is located on linkage group (LG) ‘a’, and Marker 2 is located on linkage group ‘b’. Marker 2 was detected only after the genotypes for Marker 1 were included as a fixed effect in the one QTL model for QTL Express.
CHAPTER 8: GENERAL CONCLUSIONS
8.1 Synopsis

To aide the study of human complex disease, the development and validation of appropriate biomedical models is important (reviewed in Leader and Padgett, 1980; reviewed in Swansen, et al., 2004). The human idiopathic-type scoliosis syndrome (IS) is a complex phenotype for which an animal model is needed in order to study the aetiology and pathogenesis of curvature. The aim of this thesis is to build the curveback guppy lineage into a tool for understanding the biology of idiopathic-type spinal curvature. In sum, the chapters in this thesis demonstrate that as a model, the curveback guppy can provide insight into not only the etiopathogenesis of IS, but also risk factors for severe curvature.

The content of chapters two through six is focused on validation of the curveback model through the articulation of unique phenotypic parallels to IS, and chapter seven describes initial efforts to map genes that are associated with curvature. Considering that the genetic basis of IS remains unknown, it is important to identify genes that are associated with curveback in order to define the biology of the deformity and provide candidates for human studies. The work described in this thesis is meant to provide a foundation for further research into biological aspects of idiopathic-type scoliosis.

8.2 Summary of results and new research potentials

Prior to the work introduced in this thesis, idiopathic-type spinal curvature was considered a deformity that occurs exclusively in humans. This notion is supported by the fact that prior to the guppy, no animal has ever been demonstrated to have idiopathic-type spinal curvature. In chapter four I explore
how the history of animal models used for study has fostered the notion that IS is exclusive to bipedalism. Teleosts have never been considered for the study of human spinal deformity. In chapter two, I review spinal curvature among teleosts, and propose that they can be of value to biomedical research because they are tractable, have a diversity of non-induced vertebral deformities, and substantial genomic resources. I suggest that, as models for understanding the basic elements of vertebral stability, deformity, and genetics, teleosts are an unexploited and highly useful resource.

In chapter three I characterize the curveback phenotype morphologically, developmentally, and genetically so that it can be compared to IS. In each of these categories, I describe unique phenotypic parallels between curveback and human idiopathic-type spinal curvatures and suggest that curveback is a feasible model for the study of biological factors involved in the aetiology of IS. This general assessment of the curveback phenotype is meant to encourage further research into the morphology, development, and genetics of curveback.

Accordingly, chapter five characterizes morphological changes that are associated with the vertebrae involved in curvature, chapter six investigates the length of the tails in proportion to the body in fish with curvature, and chapter seven describes chromosomal regions that are associated with curvature. In addition to providing further support for the feasibility of curveback as a model for IS, these studies raise questions that encourage further research.

The distortion of vertebral shape is an important phenotypic parallel to human IS that occurs despite ontological differences between human and teleost
vertebrae. *Curveback* is the first animal to demonstrate non-induced distortion of vertebrae in association with spinal curvature. The results from chapter five suggest that structural and microarchitectural changes in affected vertebrae and intervertebral regions are an adaptive response to an unknown force. With both *curveback* and IS curves, these adaptations reflect asymmetric cranio-caudal loading along the spine and a greater force acting on the concave side of curvature. To further explore this non-induced aspect of the phenotype in *curveback*, histochemical changes should be surveyed. For example, the activity of skeletal proteins such as osteocalcin in distorted and non-distorted vertebrae in curved guppies as well as in normal guppies could be investigated. Also, efforts should be made to investigate the integrity of collagen in normal and curved individuals to rule-out the possibility that the cause of scoliosis in *curveback* is due to a deficiency of lysyl hydroxylase. In humans, Ehlers-Danlos syndrome type IV is a rare autosomal recessive disorder that is caused by a deficiency of lysyl hydroxylase and scoliosis is a symptom.

Results from chapter six show that although the absolute length of a fish does not correlate to curvature, among adult curved females, the length of the tail is disproportionately long compared to the length of the body. This length disproportion is positively correlated to curve magnitude, and females with extreme length disproportion exhibit an apparent growth abnormality. These results are similar to human anthropometric studies that have found disproportionate body lengths among girls with IS. Further experiments using *curveback* should characterize the lengths of the body and tail in males and
females throughout growth to see if there are differences between genders. Also, the individual lengths of vertebral bodies for curved and non-curved fish should be measured to see if all vertebrae are longer, or only some vertebrae.

Chapter seven focuses on efforts to map the QTL that are associated with idiopathic-type spinal curvature in *curveback*. I generated 286 F2 and 129 backcross mapping progeny. Using guppy-specific SNP markers on 84 backcross offspring we identified two linkage groups that are significantly linked to curvature and explain an estimated 44.6% of the total phenotypic variance. Further genetic study involves the identification of candidate genes in these loci. Candidate genes will be tested on the *curveback* pedigree to see if there are more than one predisposing major gene segregating in the population; other teleost species; human pedigrees; and quadrupedal animals. In addition to mapping candidate genes for curve predisposition, important modifier genes that might explain phenotypic variation observed in the *curveback* mapping crosses should be identified.

The *curveback* pedigree described in chapter one serves as a resource for further study. Importantly, the fact that selection for the highest curve magnitude has evolved the *curveback* phenotype into a three-dimensional deformity requires attention, as does the fact that some curves resolve to normal or nearly normal before maturity. Selection has demonstrated that these components are genetic, and so future studies will attempt to explain the genetic basis. Also, further morphological study should investigate the relationship between vertebral shape distortion and curve pathogenesis in resolving, two-dimensional, and three-
dimensional curves. Technology such as in-vivo micro-CT imaging would allow for investigation into a correlation between growth, vertebral shape, and a final adult phenotype.

8.3 Contribution to basic and medical sciences

The study of how idiopathic-type spinal curvature arises in the curveback guppy model is an important basic science pursuit that may lead to a greater understanding of molecular and cellular mechanisms involved in spinal curvature. Generally, the genetic factors that maintain spinal integrity are not well understood. The importance of such an understanding is demonstrated by the fact that US National Institute of Health (NIH) has given priority to research regarding the genetics and underlying biological processes for various types of bone disease and skeletal deformities (2007 until present; Congressional Appropriations Bill, House Report 109-515). The growing burden of musculoskeletal disorders on society has prompted the United States President to declare 2002-2011 the 'Bone and Joint Decade (USBJD)', in order to raise awareness of this burden, enhance collaborative efforts among individuals and organizations, and advance research that will lead to improvements in prevention, diagnosis, and treatment (American Academy of Orthopaedic Surgeons, 2008).

Characterization of phenotypic parallels between human and curveback idiopathic-type scoliosis phenotypes is important for the establishment of curveback as a relevant biomedical model. Exploration of these phenotypic parallels and identification of genes involved in the curveback model has the potential to elucidate important biological pathways involved in idiopathic-type
curvature. This research should help to identify biological pathways that are involved in the human idiopathic-type scoliosis (IS) syndrome, that will ideally lead to the identification causative genes, leading to more effective screening and early curve detection, and suggest possible therapeutic interventions.

8.4 Literature Cited

