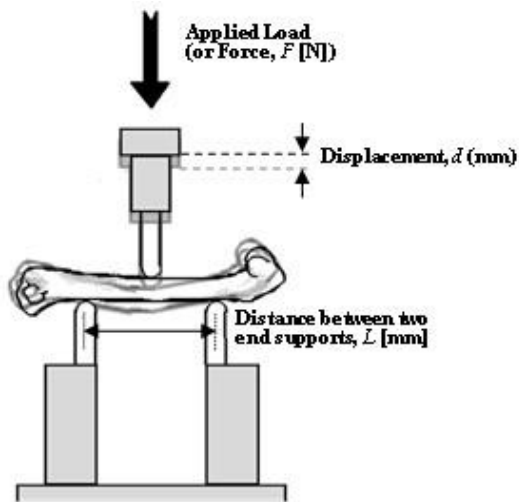


## Bone Mechanical Testing by Three-Point Bending

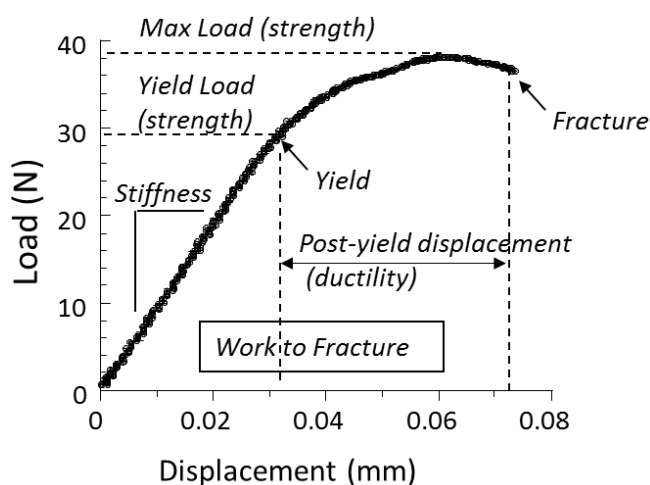
In a three-point bending test, the whole bone is placed in a fixture attached to a materials testing machine and loaded until broken (Fig. 1). This is a structural mechanical test because it tests the properties of the “whole bone” as a structure. More precisely, it tests the mechanical properties of the mid-diaphysis, which is typically all cortical bone. It is appropriate for testing femurs or other long bones. At the start of the test, the bone is in an undeformed state (zero displacement) and the loading point makes contact with the bone with a small pre-load ( $< 1$  N) in order to keep the bone in place. The loading point then moves progressively downward with increasing applied load (or force,  $F$ ) and displacement ( $d$ ). Load and displacement values are recorded during the test and stored in a data file. From these data we directly determine whole-bone (structural) mechanical properties (section I). We then combine these properties with the bone geometric properties from microCT to estimate bone material properties (section II). For this reason we do microCT scanning prior to mechanical testing of the same bones.



**Figure 1.** Schematic diagram of a three-point bending test. Dark lines depict the undeformed bone, and the lighter lines the deformed (displaced) bone. The bone is placed on two support points, and a third (loading) point applies a downward load at the mid-diaphysis. For femur testing (as shown) we put the anterior surface down so the posterior aspect of the femoral condyles faces up. Failure typically occurs at or near the loading site when a crack forms on the anterior surface (tensile side) opposite the loading point, and propagates across the bone to the posterior surface (compressive side).

### I. Whole-Bone Mechanical Properties

A plot of the load vs. displacement data is generated for analysis (Fig. 2). From the load-displacement plot, we derive a number of parameters that describe the structural properties. The five parameters recommended for a basic description are: stiffness, yield load, ultimate load, post-yield displacement and work-to-fracture (Table 1).



**Figure 2.** Example load-displacement plot from a mouse femur bending test. The portion of the curve to the left of the yield point represents the elastic behavior of the bone. If you release the load before reaching the yield point, the bone springs back to its original shape like an elastic band. To the right of the yield point represents the post-yield (or plastic) behavior. If you load past the yield point the bone will no longer spring back to its initial shape but will be permanently deformed (damaged). Past the yield point the load increases to its maximum value. The final event of the test is fracture, when the load drops sharply toward zero.

Nomenclature: The terms ‘load’ and ‘force’ are used interchangeably. Historically we have used ‘force ( $F$ )’, but in keeping with a recent review article <sup>(1)</sup>, we will adopt the term ‘load’ as our standard. This is strictly a nomenclature issue and does not affect interpretation.

**Table 1.** Standard whole-bone mechanical properties derived from a three-point bending test.

<b>“Standard” nomenclature per Jepsen et al. <sup>(1)</sup> [units]</b>	<b>“Alternative” nomenclature [units]</b>
Stiffness [N/mm]	<i>same</i>
Yield Load [N]	Yield Force [N]
Maximum Load [N]	Ultimate Force [N]
Post-Yield Displacement (PYD) [mm]	<i>same</i>
Work-to-Fracture [Nmm]	Energy-to-Fracture [Nmm]

**What do these properties mean?**

**Stiffness** is defined as the slope ( $\Delta y/\Delta x$ ) of the linear region of the load-displacement curve. It is a measure of the resistance offered by the whole bone to the applied displacement during the elastic region and is analogous to a simple spring constant (K) from physics. The stiffer a bone is the more force it takes to produce a given displacement, and hence a steeper slope. **Yield load** indicates the value of load at the yield point, which is where the load-displacement plot deviates from linearity. It is a measure of how much load the bone can sustain before it suffers permanent damage. It is one measure of a bone’s strength. **Maximum load** (aka, Ultimate Force) is simply the maximum value of load attained during the test. It is the simplest measure of the whole bone’s strength. The stronger a bone is, the higher its maximum load. **Post-yield displacement (PYD)** is the displacement from the yield point to the fracture point, and is a measure of ductility. A bone with high post-yield displacement is said to be ‘ductile’ and sustains a lot of damage before fracture. Bones with very little post-yield displacement are ‘brittle’ (chalk-like). **Work-to-fracture** (aka, Energy-to-fracture) is defined as the total area under the curve. It represents the work that must be done to fracture the bone, sometimes called the whole-bone toughness. A tough bone requires more work to fracture. Historical data are presented below (Appendix) for reference purposes.

**Are these properties inter-related?**

Each property in Table 1 tells you something specific about whole-bone behavior. Nonetheless, under normal conditions the properties are not all independent, i.e., some are correlated. Stiffness, yield load and max. load are often highly correlated because they all vary in similar way with underlying bone traits (next section). Bones with high stiffness tend to be strong. Post-yield displacement is independent of the above. Work-to-fracture is influenced both by PYD and max load, and thus may correlate with either or both.

**What other bone traits influence whole-bone mechanical properties?**

The two types of traits that influence whole-bone properties are: 1) morphology, 2) material properties. Mathematically, this can be expressed as:

$$\text{Structural Properties} = f(\text{Morphology, Material})$$

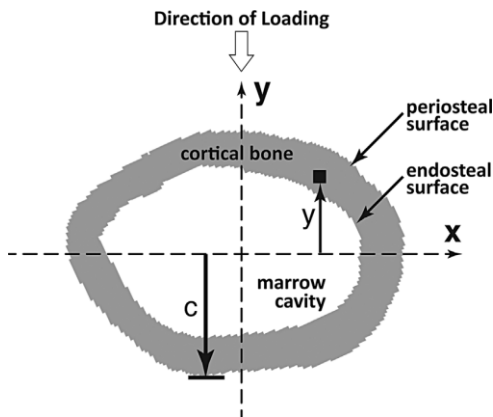
Related to morphology, a bone with a larger diameter (width) at the mid-diaphysis will tend to be stiffer and stronger. Thus, if one of your experimental groups has increased bone area, you can expect that this will lead to increased stiffness and max. load. The larger bone may not have increased PYD, as this property tends to be more dependent on material. In terms of material properties, if the diaphysis is made of cortical bone that has high material strength, it will be stronger at the whole-bone level. This is explained further in Section II.

**Does the geometry of the test fixture affect the properties?**

Yes! Each of the values in Table 1 depends on the distance between the supports (called the support span length, ‘L’ in Figure 1). Increasing span length will tend to result in lower values of stiffness, yield and max. load, while increasing PYD. This only matters if: 1) the span length is varied between samples in your study, or 2) you are making comparisons between two studies done using different span lengths (or comparing 3- vs. 4-point bending data). This is why we do not often vary span length (L = 7 mm is our standard for femur). Note that there are ways to normalize values to account for the effect of span length; for more information, refer to Jepsen et al., Supporting Information, Section 1.<sup>(1)</sup> Historically, Silva lab papers have presented results in terms of these normalized values<sup>(2)</sup>.

## II. Estimated Material Properties

**Diaphyseal Morphology.** Diaphyseal (cortical) morphology should have already been obtained through prior microCT analysis (Fig. 3). You will have determined the bone area (BA), medullary area (MA), total area (TA = BA + MA), cortical thickness (Ct.Th) and polar moment of inertia (pMOI). To compute the estimated material properties you will need two additional parameters:  $I_{min}$  and  $c$ . These can be obtained using a custom MATLAB script.  $I_{min}$  is the minimum value of moment of inertia for the bone, and corresponds to the orientation that provides the least resistance to bending; this should match up with the orientation during the bending test. ' $c$ ' is defined in Figure 3.



**Figure 3.** Idealized cross-section of a mouse mid-shaft illustrating the outer (periosteal) surface, inner (endocortical) surface, marrow cavity, and solid cortical bone. To quantify morphology, each bone cross-section is discretized into pixels (square), each with a known distance from the bending axis (eg, the distance  $y$  shown beneath the square). ' $c$ ' is the perpendicular distance from the neutral axis to the furthest point on the tensile surface, where failure is likely to initiate.

**Material Properties\*.** We then calculate material properties that are analogous to the whole-bone mechanical properties, except that they are normalized to account for bone size (Table 2). These properties represent the behavior of a single 'unit' of cortical bone, like a cube that is 1 mm x 1 mm x 1 mm. They are sometimes called 'tissue-level properties'. The three properties we report from this analysis are: **elastic modulus** (also called Young's modulus), **yield stress** and **ultimate stress**.

**Table 2.** Standard material properties estimated from whole-bone properties and morphology using engineering beam-theory equations.

Whole-bone mechanical properties [units]	Material properties [units]	Equation
Stiffness [N/mm]	Elastic modulus, $E$ [N/mm <sup>2</sup> = MPa]	$E = KL^3/(48 I_{min})$
Yield Load [N]	Yield Stress, $\sigma_y$ [N/mm <sup>2</sup> = MPa]	$\sigma_y = F_yLc/(4 I_{min})$
Maximum Load [N]	Ultimate Stress, $\sigma_{ult}$ [N/mm <sup>2</sup> = MPa]	$\sigma_{ult} = F_{ult}Lc/(4 I_{min})$

Parameters are as shown in Figs. 2 and 3.  $K$  = stiffness,  $L$  = span length,  $I_{min}$  = min. moment of inertia,  $F_y$  = yield force (yield load),  $F_{ult}$  = ultimate force (max. load),  $c$  = distance from centroid of cross-section to outermost point on the cross-section, which can be approximated as bone width/2.

**\*Caveat:** The methods described herein will provide estimates of material properties that are inherently inaccurate for samples the size of mouse bones. But, if the same methods are used across all your samples you should obtain reliable relative values. These values are useful as a "first pass" analysis of material properties; if they show differences between experimental groups, more sophisticated analyses may be warranted.

### References

1. Jepsen KJ, Silva MJ, Vashishth D, Guo XE, van der Meulen MC 2015 Establishing biomechanical mechanisms in mouse models: practical guidelines for systematically evaluating phenotypic changes in the diaphyses of long bones. *J Bone Miner Res* 30(6):951-66. *+Important Reference+*
2. Brodt MD, Ellis CB, Silva MJ 1999 Growing C57Bl/6 mice increase whole bone mechanical properties by increasing geometric and material properties. *J Bone Miner Res* 14(12):2159-2166.

**APPENDIX: HISTORICAL DATA**

**Femur three-point bending.** Data from female and male Balb/C WT mice at 2, 4, 7, 12 and 20 months age (mean  $\pm$  SD; n = 8-11/group). From Willingham et al. (support span = 7 mm)

Age (mo)	Female					Male				
	2	4	7	12	20	2	4	7	12	20
Stiffness [N/mm]	78 $\pm$ 7.0	123 $\pm$ 23	136 $\pm$ 22	166 $\pm$ 22	158 $\pm$ 23	89 $\pm$ 13	127 $\pm$ 13	164 $\pm$ 25	168 $\pm$ 38	184 $\pm$ 26
Yield Load [N]	11.2 $\pm$ 0.8	15.0 $\pm$ 1.0	17.2 $\pm$ 2.4	19.3 $\pm$ 2.6	16.5 $\pm$ 2.6	12.2 $\pm$ 1.3	16.8 $\pm$ 1.8	19.1 $\pm$ 4.6	20.1 $\pm$ 3.8	17.9 $\pm$ 3.4
Max Load [N]	13.5 $\pm$ 0.8	20.1 $\pm$ 3.4	24.1 $\pm$ 3.5	29.2 $\pm$ 4.0	23.0 $\pm$ 4.2	15.1 $\pm$ 1.8	21.7 $\pm$ 1.6	26.6 $\pm$ 4.9	25.6 $\pm$ 3.1	25.7 $\pm$ 4.2
Post-Yield Displacement [mm]	0.27 $\pm$ 0.07	0.21 $\pm$ 0.05	0.19 $\pm$ 0.06	0.18 $\pm$ 0.05	0.08 $\pm$ 0.03	0.40 $\pm$ 0.19	0.22 $\pm$ 0.07	0.19 $\pm$ 0.06	0.13 $\pm$ 0.05	0.15 $\pm$ 0.07
Work-to-Fracture [Nmm]	4.2 $\pm$ 0.7	5.0 $\pm$ 0.8	5.2 $\pm$ 1.0	6.0 $\pm$ 1.6	2.6 $\pm$ 0.9	6.1 $\pm$ 2.0	5.8 $\pm$ 1.5	6.0 $\pm$ 2.0	4.6 $\pm$ 1.3	4.4 $\pm$ 1.2

**Femur diaphyseal morphology.** Data from female and male Balb/C WT mice at 2, 4, 7, 12 and 20 months age (mean  $\pm$  SD; n = 8-11/group). From Willingham et al.

Age (mo)	Female					Male				
	2	4	7	12	20	2	4	7	12	20
Bone Area (BA) [mm <sup>2</sup> ]	0.72 $\pm$ 0.02	0.89 $\pm$ 0.07	0.98 $\pm$ 0.05	1.06 $\pm$ 0.09	1.08 $\pm$ 0.03	0.79 $\pm$ 0.06	0.95 $\pm$ 0.04	1.06 $\pm$ 0.12	1.03 $\pm$ 0.05	1.02 $\pm$ 0.09
Cortical Thickness (Ct.Th) [mm]	0.230 $\pm$ 0.015	0.271 $\pm$ 0.017	0.285 $\pm$ 0.013	0.301 $\pm$ 0.025	0.275 $\pm$ 0.016	0.257 $\pm$ 0.033	0.294 $\pm$ 0.011	0.335 $\pm$ 0.039	0.317 $\pm$ 0.032	0.317 $\pm$ 0.034
Ixx [mm <sup>4</sup> ]	0.075 $\pm$ 0.005	0.105 $\pm$ 0.018	0.122 $\pm$ 0.014	0.153 $\pm$ 0.027	0.201 $\pm$ 0.013	0.093 $\pm$ 0.010	0.107 $\pm$ 0.010	0.129 $\pm$ 0.033	0.141 $\pm$ 0.007	0.153 $\pm$ 0.021

**Femur material properties morphology.** Data from female and male Balb/C WT mice at 2, 4, 7, 12 and 20 months age (mean  $\pm$  SD; n = 8-11/group). From Willingham et al.

Age (mo)	Female					Male				
	2	4	7	12	20	2	4	7	12	20
Elastic Modulus (E) [MPa]	7400 $\pm$ 800	8400 $\pm$ 900	8000 $\pm$ 1000	7800 $\pm$ 500	5600 $\pm$ 900	6800 $\pm$ 600	8500 $\pm$ 1100	9200 $\pm$ 1200	8500 $\pm$ 1700	8700 $\pm$ 1700
Yield Stress ( $\sigma_y$ ) [MPa]	116 $\pm$ 9	124 $\pm$ 12	127 $\pm$ 17	123 $\pm$ 12	89 $\pm$ 16	130 $\pm$ 8	136 $\pm$ 21	160 $\pm$ 20	157 $\pm$ 31	136 $\pm$ 29
Ultimate Stress ( $\sigma_{ULT}$ ) [MPa]	140 $\pm$ 11	163 $\pm$ 9	178 $\pm$ 21	186 $\pm$ 16	124 $\pm$ 26	161 $\pm$ 11	174 $\pm$ 13	218 $\pm$ 18	199 $\pm$ 23	194 $\pm$ 30

Reference: Willingham MD, Brodt MD, Lee KL, Stephens AL, Ye J, Silva MJ 2010 Age-related changes in bone structure and strength in female and male BALB/c mice. Calcif Tissue Int 86(6):470-83.