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significant differences in ash content or collagen content were detected, however. These data suggest that GDF-7 deficiency is associated with elevated cortical bone material properties, which compensate for decreased geometric properties, thereby preserving bone structural integrity. The compositional and/or microstructural bases for these altered material properties remain to be determined, however.

GEOMETRIC AND MATERIAL CONTRIBUTIONS TO WHOLE BONE STRUCTURAL BEHAVIOR IN GDF-7 DEFICIENT MICE

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SUMMARY:

The growth/differentiation factors (GDFs) are a subfamily of bone morphogenetic proteins (BMPs) known to play a role in a variety of skeletal processes. Previous work using the *brachypod* mouse demonstrated that mice deficient in GDF-5 have long bones with diminished material properties and ash content compared to control littermates. The aim of the present study was to examine the role of a related GDF family member, GDF-7 (BMP-12) in cortical bone by examining the geometric and material contributions to whole bone structural behavior in GDF-7 deficient mice. Femora from 16 week old GDF-7 -/- animals had significantly smaller bone cross sectional geometric parameters (e.g. –20% medial/lateral and anterior/posterior moments of inertia). Despite having smaller bone cross-sections, all structural parameters obtained from four-point bending tests were comparable to those of wild type bones due to elevated cortical bone material properties (+18% modulus of elasticity, +28% yield strength, and +18% ultimate strength). No significant differences in ash content or collagen content were detected, however. These data suggest that GDF-7 deficiency is associated with elevated cortical bone material properties, which compensate for decreased geometric properties, thereby preserving bone structural integrity. The compositional and/or microstructural bases for these altered material properties remain to be determined, however.

KEY WORDS:

GDF-7, BMP-12, bone, material properties, bone biomechanics

INTRODUCTION:

The Growth/Differentiation Factors (GDFs) are a subset of the Bone Morphogenetic Proteins (BMPs) known to be involved in numerous skeletal processes, including joint formation and endochondral bone growth [1-3]. Deficiencies of BMPs and GDFs cause a variety of skeletal abnormalities in bone as well as tendon [4]. While deficiencies in BMP family members have been shown to affect cortical bone mechanical behavior [5,6], little is known regarding the role of GDFs in determining bone material properties. Recently, it has been demonstrated that deficiency in GDF-5 may adversely affect the structural behavior of mouse long bones through a reduction in material properties [7].

GDF-7 (*a.k.a.* BMP-12) is a protein closely related to GDF-5 [8], but which has been the subject of much less investigation. GDF-7 has been shown to affect connective tissue ultrastructure, as mice deficient in this factor have tendons with smaller type I collagen fibrils [9]. Additional studies suggest that BMP-12/GDF-7 may be involved in tendon formation [3,10] and/or repair [11]. In the present study, we were interested in examining the effect of GDF-7 deficiency on the biomechanical behavior of another type I collagen rich tissue: cortical bone. Specifically, we were interested in determining whether GDF-7 deficiency affects the structural behavior of murine cortical bone and, if so, what the basis for these differences might be (i.e. geometric and/or material property differences). A secondary hypothesis was that gender would modulate the effect of genotype on cortical bone geometry, structural, and/or material behavior.

METHODS:

Experimental Model

Experimental animals used for this study consisted of 16-week-old male and female mice deficient in GDF7 (-/-) and their wild type (+/+) littermates obtained from intercrossing heterozygous carriers of the targeted GDF-7 knockout on a C57BL/6-J x 129SV/J background. Knockouts had been generated by replacing a 1.1 kb fragment containing the mature signaling domain of the GDF-7 gene with a positive selectable neomycin resistance cassette [12]. A total of ten mice in each of four groups (male and female, -/- and +/+) were used for analysis. Animals were sacrificed via CO_2 inhalation in accordance with IACUC guidelines. Immediately following sacrifice, animals were weighed, and body mass recorded. Bones were dissected free from soft tissue, lengths measured in duplicate using sliding digital calipers, then wrapped in saline soaked gauze, and frozen at –20°C in hermetically sealed bags until needed. Average values of duplicate length measurements were taken as the representative length for a given bone, and averages of left and right sides were taken as the average femoral and tibial lengths for a given animal. For each animal, the left femur was used for mechanical testing in four-point bending, the right femur was used for determination of cross-sectional geometric properties, left tibia for ash content, and right tibia for collagen content as indicated by hydroxyproline analysis.

Cross-sectional Geometry

The right femur was embedded in polyester resin. Serial cross-sections were made through the bone diaphysis using a circular diamond-blade saw (Leica SP1600, Leica

Microsystems, Bannockburn, IL). 100μm thick sections were obtained, stained with a von Kossa stain, imaged and captured using a transmission light microscope (Olympus BX51, Olympus America, Melville, NY) and digital camera (Olympus DP70, Olympus America, Melville, NY). The section immediately distal to the third trochanter was then analyzed with the FORTRAN program VA-TWIST [13] to determine cortical bone area and moments of inertia about the medial/lateral and anterior/posterior axes (I_{ml} and I_{ap}).

Mechanical Testing

Left femora were mechanically tested to failure in four-point bending using the protocol described by Brodt et al. [14] on an Instron materials testing system [Model 5542, Instron Corp., Canton, MA). Prior to testing, bones were thawed and soaked for 3h in PBS at room temperature to ensure hydration. Bones were loaded by applying displacement to the posterior side at a rate of 0.03 mm/s, thereby creating a bending moment about the medial/lateral axis. To allow comparison of bones with different lengths, displacement data were normalized by the quantity $(3aL - 4a^2)/6$ where L is the distance between the (lower) support points (10 mm) and a is the distance between the (upper) loading and (lower) support points (2 mm). The following structural properties were then calculated from the Moment (i.e. force x $a/2$) vs. normalized displacement curves: (1) ultimate moment (M_{ult}); (2) bending rigidity; (3) ultimate displacement; (4) ultimate bending energy; (5) yield moment (M_y); and (6) yield displacement. Estimates of the following material properties were calculated from structural and cross-sectional geometric properties using the equations:

$$\text{Ultimate Strength} = \sigma_{\text{ult}} = \frac{M_{ult} \times y_{\max}}{I_{ml}} \quad (1)$$

$$\text{Yield Strength} = \sigma_{y} = \frac{M_{y} \times y_{\max}}{I_{ml}} \quad (2)$$

$$\text{Young's Modulus} = E = \frac{\text{bending rigidity}}{I_{ml}} \quad (3)$$

where y_{max} is the distance from the section centroidal (medial/lateral) axis to the outermost periosteal fiber on the tensile (anterior) aspect of the section. Yield Strain (ε_y) was calculated via Hooke's Law by dividing Yield Strength by Young's Modulus. Data from 2 -/- females, 2 -/-males, 1 +/+ female, and 2 +/+ male bones were discarded due to deviations in testing protocol, thus leaving a total of 16 mutant bones (8 male, 8 female) and 17 wild type bones (8 male, 9 female) for analysis of structural and material properties.

Ash Content

The left tibia was processed for determination of ash fraction. Proximal and distal regions of the bone were cut away to allow marrow to be removed via PBS. The dry mass of each tibia was obtained after defatting in acetone for 72 hours (replacing the acetone solution every 24 hours), air drying for 24 hours, and drying at 60^{o}C for six hours [15]. Ash weight was determined after heating for 18 hours at 600^{o}C. Ash content was then calculated as (ash mass/dry mass) x 100.

Collagen Content

After acid hydrolysis in 6N HCl for 18 hours at 110°C, the collagen contents of the right tibiae were assessed by measuring the amount of hydroxyproline per unit dry weight. The dimethylaminobenzaldehyde (DMBA) colorimetric assay for hydroxyproline was used [16]. All samples and standards were processed in duplicate and values averaged for each pair.

Data Analysis

All geometric, structural and material parameters were analyzed using a two-factor ANOVA with genotype (-/-, +/+) and gender (M, F) as the independent variables (Statview, SAS Institute Inc., Cary, NC). Because the interaction between gene and gender was not statistically significant for any of the dependent variables examined, males and females were pooled to examine the effect of genotype alone. A statistical significance level of $p < 0.05$ was used.

RESULTS:

GDF-7 deficient mice were smaller in body mass than wild type littermates, but not significantly so (-7%, p = 0.1457). Both femur and tibia lengths were significantly shorter in mutant animals, although the magnitude of these differences was very small (-2%, p = 0.004) (Table 1).

Cross-sectional Geometry

Compared with wild type bones, GDF-7 deficient femora had significantly less cortical bone (-10%, p = 0.0166), and significantly smaller moments of inertia about both the anterior/posterior and medial/lateral planes (-22%, p = 0.0049 and –20%, p = 0.0032,

respectively) (Figures 1 and 2). When cross-sectional geometric properties were normalized to body mass, both normalized moments of inertia were significantly lower in GDF-7 deficient mice (-17% $p = 0.0042$ for I_{ap}/BM and –17% $p = 0.0067$ for I_{ml}/BM), thus suggesting that the cross-sections of mutant femora were smaller than expected for animal body mass (Figure 2).

Structural Properties

Despite having smaller cross-sectional geometric properties, GDF-7 deficient femora withstood comparable bending moments at yield and failure, had comparable bending rigidity and ultimate bending energy, as well as yield and ultimate displacements when compared to wild type bones (Table 2).

Material Properties

Material properties obtained by combining structural data from the left femur with geometric data from the right femur demonstrated that GDF-7 deficiency was associated with a significantly higher Young's Modulus of Elasticity (+18%, $p = 0.0409$), Yield Strength (+22%, $p = 0.0249$) , and Ultimate Strength (+18%, $p = 0.0258$) (Figure 3). Yield Strain was comparable between mutants and controls ($p = 0.72411$; Figure 3).

Composition

GDF-7 deficiency did not significantly affect either of the cortical bone compositional parameters examined: ash content was approximately 73% in both mutant and control

bones, and hydroxyproline content (indicative of collagen) was approximately 2% in both groups (Table 3).

DISCUSSION:

This study shows that 16-week-old GDF-7 deficient mice have comparable femoral whole bone structural properties compared to wild type littermates despite having significantly smaller bone cross-sectional geometric properties. Comparable structural integrity is accomplished via elevated material properties, although the compositional and/or ultrastructural bases for these material differences remains to be determined: neither ash content nor collagen content was significantly different between mutants and controls. Gender did not modulate the effect of genotype on any of the bone properties examined.

Is the primary effect of GDF-7 deficiency on bone geometry (with secondary material adaptation), or on material properties (with secondary geometric adaptation)? One could argue that since bone size is *inappropriate* for animal body mass in GDF-7 deficient mice (Figure 2), mutant bones may have adapted poorly to their local mechanical loading environment, as mechanical stimulus is likely to be proportional to animal body mass. A more careful analysis, however, would consider bone strain as a more relevant indicator of local stimulus for bone mechanical adaptation [17]. In the present study, no difference was apparent between the strain at yield (the last point in the linear elastic region of the bone for which Hooke's law is valid) between GDF-7 -/- and +/+ animals (Figure 3), thus suggesting that mutant bone is appropriately adapted to local mechanical stimuli. It is

therefore more likely that the primary effect of the absence of GDF-7 on bone is on material properties, with secondary geometric adaptation to maintain comparable *in vivo* strains and (additionally) whole bone structural behavior.

The underlying causes of the elevated material properties in the GDF-7 deficient femora are unclear. Bone is largely composed of two materials – mineral (hydroxyappatite) and type I collagen – and alterations in the quantity or quality of either may influence material properties. Non-collagenous proteins and cellular components comprise a much smaller, but by no means negligible, contribution to composition. Little exists in the current literature regarding cellular or tissue-level effects of GDF-7. Recently, it has been suggested that GDF-7 increases osteoblast proliferation and alkaline phosphatase expression [18], although others have failed to substantiate this finding [10,19]. Studies also indicate that other Growth/Differentiation Factors can affect mineral content of long bones [7]. Our study, however, did not find mineral content to be altered by GDF-7 deficiency, as ash content in mutant and control bones did not differ significantly. It should be noted that femora were used for mechanical testing while tibiae were used for ash content analysis. Consequently, if GDF-7 deficiency results in bone-specific effects on mineral content, differences in mineral content could be present in the femur despite the comparable levels of ash fraction found in mutant and control tibiae. It is also possible that differences in mineral quality, rather than quantity, are responsible for the observed increased material properties. Two alterations in mineral quality thought to increase bone material stiffness are increased crystal size and end-to-end appatite fusion [20-22]. Such a mechanism could be responsible for the changes seen in this study.

Investigation of the ultrastructural characteristics of mineral in GDF-7 deficient bone with FTIR (Fourier-transformed infrared microscopy) or X-ray diffraction analysis [23] would allow this possibility to be further examined.

Differences in gross collagen content also do not appear to be the basis of the increased material properties, as hydroxyproline levels did not differ between groups. This assumes, however, that hydroxyproline reflects overall collagen content equivalently in the mutants and controls, and that GDF-7 deficiency does not affect the hydroxylation of proline during collagen biosynthesis. Assuming that GDF-7 does not affect the total collagen content or hydroxylation of proline in mouse bone, it is still possible that GDF-7 could affect other characteristics of the collagen molecule and its ultrastructural organization. For example, collagen fiber directionality is an important determinant of cortical bone strength in bending tests [24]. As GDF-7 deficiency has been shown to affect fibril ultrastructure in other mouse tissues [9], it may be affecting bone in a similar fashion. Ultrastructural examination of mutant and control cortical bone might identify differences in collagen fibril size and orientation. It is also important to acknowledge the possibility that material property differences could arise due to alterations in other components of the non-collagenous organic matrix of cortical bone in GDF-7 deficient mice.

In summary, the results of this study demonstrate for the first time that GDF-7 deficiency in 16-week old mice is associated with significantly smaller bone cross-sectional properties, but comparable whole bone structural (four-point bending) behavior due to

elevated material properties. No modulation of this effect by gender was observed. Elucidating the underlying basis or bases for these observed differences in material behavior will require additional investigations into cortical bone composition and ultrastructure.

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Table 1

Table 1: Morphometric Measurements [mean (SD)][a]

	GDF7 -/- (n = 20)	GDF7 +/+ (n = 20)	Percent Difference	p-value
Body Mass (g)	25.0 (4.4)	26.9 (2.8)	-7%	0.1457
Femur Length (mm)	15.2 (0.41)	15.5 (0.40)	-2%	**0.0040**
Tibia Length (mm)	17.1 (0.51)	17.5 (0.31)	-2%	**0.0044**

[a] $p < 0.05$ *gene x gender*, thus males and females were pooled for analysis

Table 2

Table 2: Femoral Structural Properties [mean (SD)][a]

	GDF7 -/- (n = 16)	GDF7 +/+ (n = 17)	Percent Difference	p-value
Ultimate moment, M_{ult} (Nmm)	40.3 (6.8)	42.9 (11.1)	-6%	0.4260
Yield moment, M_y (Nmm)	36.5 (7.1)	37.7 (11.1)	-3%	0.7059
Bending Rigidity (Nmm^2)	1823 (570)	1915 (460)	-5%	0.6084
Ultimate displacement (mm/mm^2)	0.0312 (0.0096)	0.0304 (0.0087)	+3%	0.7939
Yield displacement (mm/mm^2)	0.0246 (0.0095)	0.0224 (0.0078)	+14%	0.4627
Ultimate bending energy (Nmm/mm^2)	0.669 (0.230)	0.744 (0.298)	-10%	0.4254

[a] $p < 0.05$ *gene x gender*, thus males and females were pooled for analysis

Table 3

Table 3: Bone Composition [mean (SD)][a]

	GDF7 -/- (n = 20)	GDF7 +/+ (n = 20)	Percent Difference	p-value
Ash Mass/Dry Mass (%)	72.7 (0.9)	72.6 (0.3)	< 1%	0.7436
Hydroxyproline/Dry Mass (%)	2.2 (0.30)	2.2 (0.31)	-	0.7548

[a] $p < 0.05$ *gene x gender*, thus males and females were pooled for analysis

FIGURE LEGENDS:

Figure 1: Representative GDF-7 -/- (left) and +/+ (right) femoral cross-sections taken immediately distal to the third trochanter and used for determination of geometric properties. Both sections are shown at the same magnification.

Figure 2: Cross-sectional geometric properties of GDF-7 -/- (white) and +/+ (stippled) femora. [a] cross-sectional area; [b] anterior/posterior moment of inertia; [c] medial/lateral moment of inertia. [d] – [f] show these same parameters normalized by animal body mass.

Figure 3: Material properties of GDF-7 -/- (white) and +/+ (stippled) femora. [a] Young's Modulus of Elasticity; [b] Yield Strength; [c] Ultimate Strength; [d] Yield Strain.

Figure 1

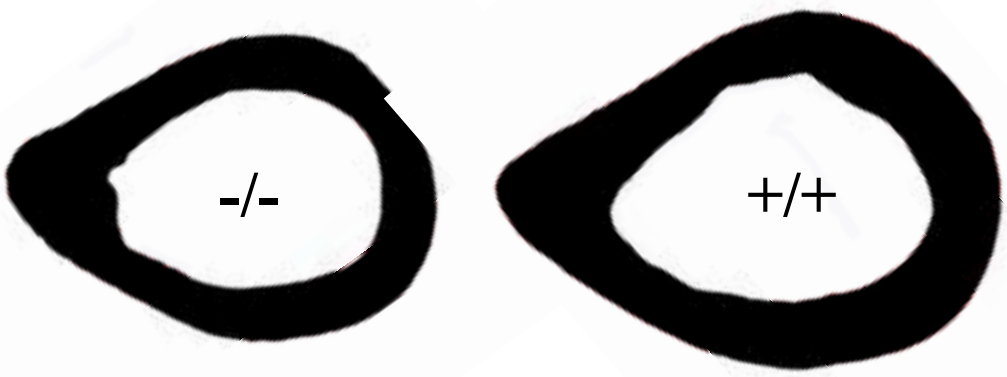


Figure 2

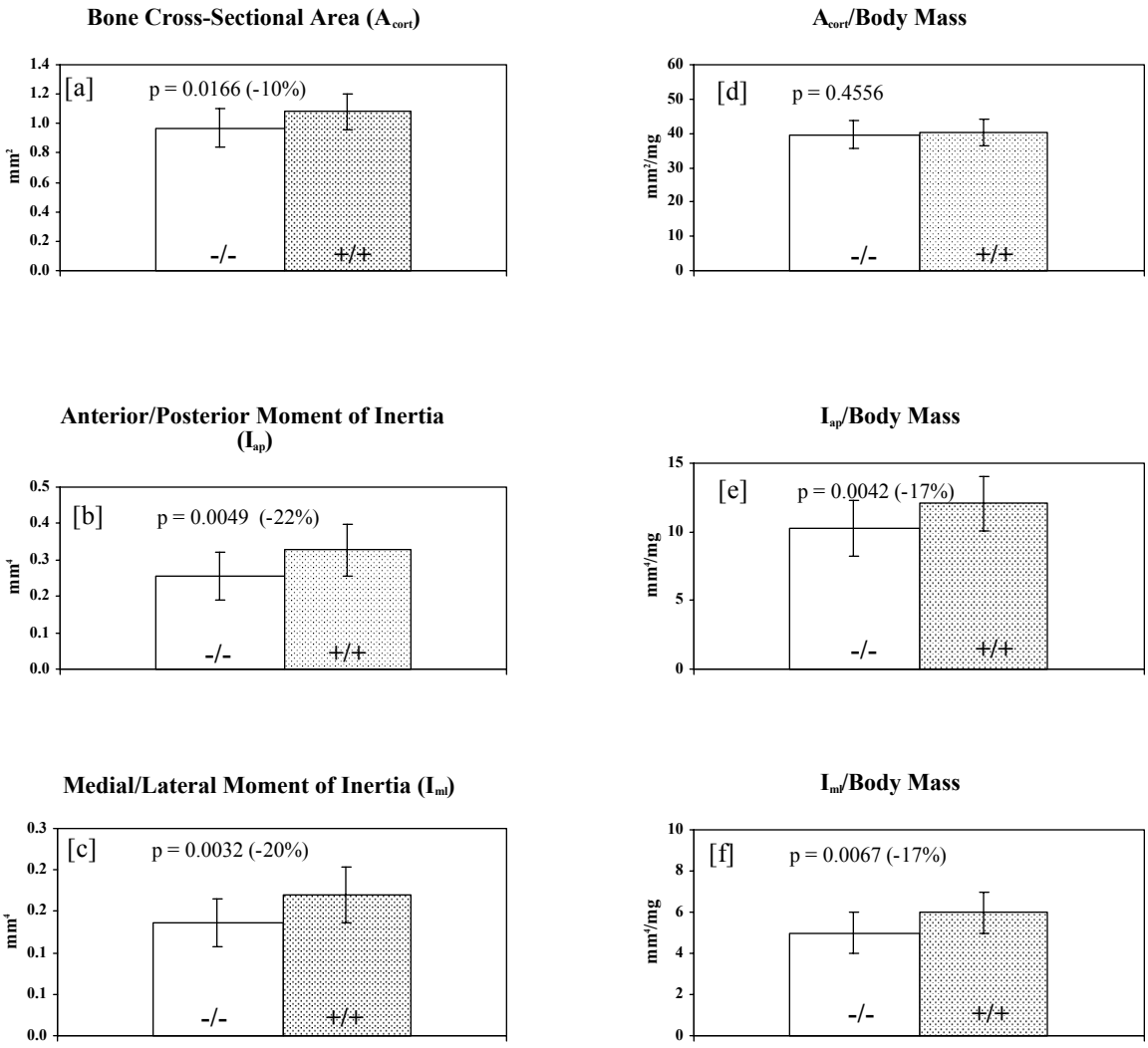
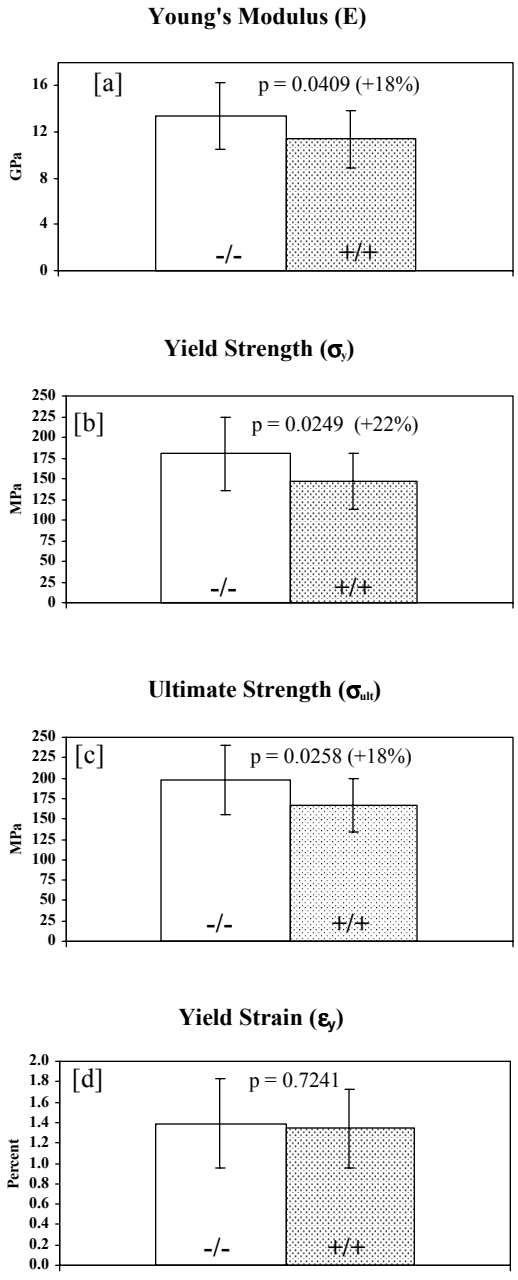

Bone Cross-Sectional Area (A_{cort})
[a]
p = 0.0166 (-10%)
mm²
-/-
+/+
A_{cort}/Body Mass
[d]
p = 0.4556
mm²/mg
-/-
+/+
Anterior/Posterior Moment of Inertia (I_{ap})
[b]
p = 0.0049 (-22%)
mm⁴
-/-
+/+
I_{ap}/Body Mass
[e]
p = 0.0042 (-17%)
mm⁴/mg
-/-
+/+
Medial/Lateral Moment of Inertia (I_{ml})
[c]
p = 0.0032 (-20%)
mm⁴
-/-
+/+
I_{ml}/Body Mass
[f]
p = 0.0067 (-17%)
mm⁴/mg
-/-
+/+

Figure 3


Young's Modulus (E)
[a]
p = 0.0409 (+18%)
GPa
16
12
8
4
0
-/-
+/+
Yield Strength (σy)
[b]
p = 0.0249 (+22%)
MPa
250
225
200
175
150
125
100
75
50
25
0
-/-
+/+
Ultimate Strength (σult)
[c]
p = 0.0258 (+18%)
MPa
250
225
200
175
150
125
100
75
50
25
0
-/-
+/+
Yield Strain (εy)
[d]
p = 0.7241
Percent
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-/-
+/+